

Binding partners for mouse acetylcholinesterase in the central nervous system

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ABBREVIATIONS

%	percentage
°C	grad Celsius
μ.	micro
aa	amino acid
AChE	Acetylcholinesterase
AD	Activation domain
ATC	acetylthiocholine iodide
ATP	adenosine triphosphate
BD	Binding domain
BChE	Butyrylcholinesterase
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
ChAT	choline acetyl transferase
CNS	central nervous system
C-terminal	At the COOH-terminus of the protein
C-terminus	COOH-terminus of the protein
DAB	3,3'-diaminobenzidine
DAPI	4,6-diamidin-2'-phenylindoldihydrochlorid
dATP	deoxyadenosine triphosphated
dCTP	deoxycytidine triphosphated
dest.	distilled
dGTP	deoxyguanosine triphosphate
dH ₂ O	distilled H ₂ O
DIG	digoxigenin
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DO-medium	Drop-out medium
dTTP	deoxythymidine triphosphate
E	embryonic day (e.g. E16)
<i>E. coli</i>	<i>Escherichia coli</i>

EDTA	ethylenedinitrilo-tetraacetic acid
e.g.	exempli gratia
<i>et al.</i>	et alteres
FCS	fetal calf serum
β-gal	β-galactosidase
g	gram
GST	glutathione-S-transferase
HEPES	4-(2-hydroxyethyl)-Piperazin-1-ethansulfonacid
kb	kilo base pair
kDa	kilo Dalton
KO	knock-out
<i>lacZ</i>	lactose catabolism gene
LB	Luria-Bretani bacterial growing mediumVII
LB agar	Luria-Bretani bacterial growing agarose
LiAc	Lithium acetate
M	molar
min	minutes
ml	milliliter
MOPS	3-(N-Morpholino)propanesulfonic acid
mm	millimeter
mRNA	messenger RNA
MW	Molecular weight
N-terminal	at the NH ₂ -terminus of a protein
N-terminus	NH ₂ -terminus of a protein
OD	optical density
o/n	over night
ORF	open reading frame
P	postnatal day (e.g. P14)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
pH	potentia hydrogenii
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature

SDS	sodium dodecyl sulfate
TAE	Tris-acetic acid-EDTA buffer
TBE	Tris-borate-EDTA
TE	Tris-EDTA-buffer
Tris	Tris-hydroxymethyl-aminomethane
U	enzymatic unit
UTR	untranslated region
UV	ultraviolet
V	Volt
Vis	Visible light
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl- β -galactosidase
YPD	Yeast extract-peptone-dextrose medium
3'	3 prime
5'	5 prime
μ l	micro liter

1. Introduction

Acetylcholinesterase (AChE) is known as the enzyme that terminates the action of the neurotransmitter acetylcholine (ACh) at cholinergic synapses. However, an increasing number of scientific works showed that AChE has functions other than the hydrolysis of ACh, related to cell differentiation, synaptogenesis, neuronal signaling and diseases. These functions are well documented, but the mechanism that supports them is not really elucidated. The aim of this thesis was to find a possible mechanism that supports developmental functions of AChE. For this it was hypothesized that AChE functions as a heterophilic cell adhesion molecule which sends signals into the neurons by binding to a membrane receptor, and searched for AChE-binding partners.

1.1. Cholinergic transmission includes a very fast and specific enzyme - AChE

AChE is a serine hydrolase and belongs to the fastest enzymes known, with the hydrolysis of ACh approaching the maximal theoretical limit set by molecular diffusion of substrate (Quinn, 1987). Acetylcholine hydrolysis can also be catalyzed by BChE, which is a close structural relative of AChE. These proteins share ~ 50% homology, concentrated in the catalytic region, but they differ in their substrate specificity (Chatonnet and Lockridge, 1989). AChE is selective towards ACh hydrolysis, which it can perform at a rate of over 10000 molecules per second. In contrast, BChE is much slower, and can also hydrolyze higher choline esters, particularly butyrylcholine. However, AChE has more functions than the ACh hydrolysis and the focus of this work is to understand one of these AChE “non-classical” functions.

Structure

Prior to 1991, the structure of AChE had been based on chemical primary sequences. Sussman and co-workers systematically cleaved amino acid sections from the C-terminus of the native protein (from Torpedo), ultimately succeeding in elucidating the crystal structure of AChE (Sussman *et al.*, 1991). This structure revealed a molecule with a α/β hydrolase fold similar to other esterases and lipases. Figure 1 shows that AChE is a globular glycoprotein, with the active site located 4 Å above the bottom of a 20 Å deep,

narrow gorge (Bourne *et al.*, 1995). The active site comprises the ``**esteratic site**``, containing the catalytic triad (Ser, His, Glu) with the active site serine responsible for hydrolysis, and the ``**anionic site**`` which binds the quaternary ammonium ion of acetylcholine (Radic *et al.*, 1997). The gorge is lined with 14 aromatic residues, forming a second or ``**peripheral anionic site**`` (PAS) (Taylor and Radic, 1994). Binding of a ligand at the PAS of AChE may cause conformational changes important in the functioning of the enzyme. PAS binds cholinesterase inhibitors selectively (Changeux, 1966; Taylor and Lappi, 1975) and these ligands can impede substrate entry to the active centre and also additionally exert an allosteric influence on the conformation of AChE (Falkenstein and Pena, 1997). It has been reported repeatedly that inhibition of this site can not only alter cholinergic activity but also modify neurite outgrowth (Bataillé *et al.*, 1998), amyloid beta association (Inestrosa *et al.*, 1996) and adhesion properties (Johnson and Moore, 1999).

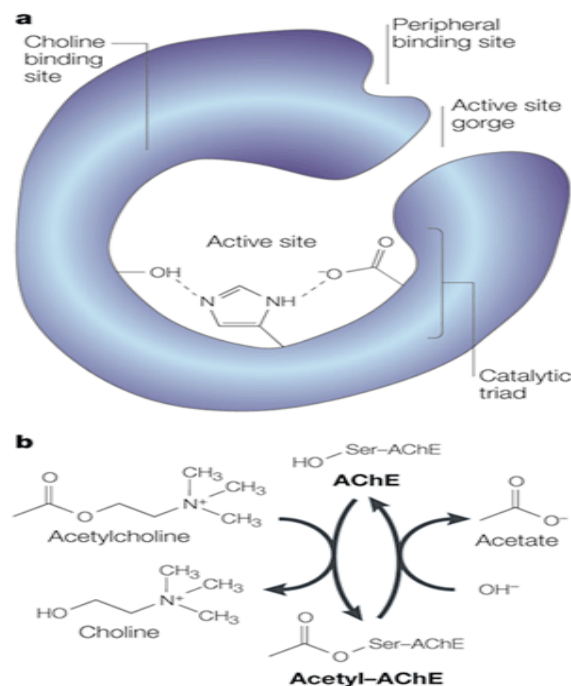
It was shown that an electrostatic dipole exists through the AChE molecule (Shafferman *et al.*, 1992a) and this can contribute to the cholinergic and non-cholinergic functions of the AChE. Electrostatic mechanisms could help to interpret the fast substrate access (Radic *et al.*, 1997) and the enzyme's role as a cell adhesion molecule (electrotactins) (Botti *et al.*, 1998).

Genetics of AChE

Mammals possess only two cholinesterase genes, an *ACHE* and a *BCHE* gene, with a single open reading frame. The mouse *ACHE* is situated on chromosome 5 (Rachinsky *et al.*, 1990; Li *et al.*, 1991, Rachinsky *et al.*, 1992). Three invariant exons of *ACHE* (exon 2, 3 and 4, with exon 1 not being transcribed) encode the signal peptide and the N-terminal 535 amino acids common to all forms of the enzyme, whereas alternative exon usage of the next exons (5 and 6) accounts for structural divergence in the C-terminus (Rachinsky *et al.*, 1990; Li *et al.*, 1991). The AChE gene generates multiple types of transcripts through a combination of different processes: the choice of several promoters; alternative splicing in the 5'-untranslated region (Luo *et al.*, 1998; Atanasova *et al.*, 1999); alternative splicing at the 3'-extremity of the coding region (Li *et al.*, 1991); the choice of several polyadenylation sites, and variable lengths of the 3'-untranslated sequence (Massoulie *et al.*, 1993). The alternative splicing generates AChE with 3 different C-terminal domains: **-H** (hydrophobic), **-R** (read-through: not spliced) and **-T** (tailed) (Massoulié *et al.*, 1998). The C-terminal domain determines the post-translational processing, quaternary organization and anchoring of the enzyme, and thus, ultimately where the enzyme will be located.

Multiple molecular forms for AChE, assembly, secretion

The two spliced variants of AChE, H and T, are homomeric and may form multiple globular subunit associations, G1, G2, G4, etc. (see Figure 2) (Massoulié *et al.*, 1993). The **R-form** does not seem to have any feature that allows for its attachment to other molecules and remains monomeric and soluble. The C-terminus of the **H-form** allows it to form disulfide-linked glycopospholipid-anchored dimers, which may be released by cleavage from the membrane. The C-terminus of the **T-form** permits diverse interactions. The AChE-T forms monomers and disulfide-bound dimers, which can be membrane-anchored or secreted in the case of proteolytic cleavage at the C-terminus.



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Figure 1. The structure of acetylcholinesterase and its catalytic reaction. (Soreq and Seidman, 2001, *Nature Neuroscience*) **(a)** Structural properties of the enzyme: note the active site, placed in a deep gorge and the peripheral anionic site. **(b)** The AChE reaction. AChE hydrolyses ACh by forming an acetyl-AChE intermediate with the release of choline, followed by the hydrolysis of the intermediate to release acetate.

Additional hydrophobic interactions allow the formation of tetramers, which can be soluble or attached to the membrane by heteromeric associations with a PRiMA anchor (proline rich membrane anchor) (Perrier *et al.*, 2002) or a collagen-like tail. The multiple molecular forms possess similar catalytic properties, but differ in their oligomeric assembly and the mode of attachment to the cell surface (see Figure 2).

There are two classes of molecular forms:

- The heteromeric species in which the catalytic units are linked to the structural units, including the asymmetric forms in which 8-12 hydrophilic catalytic subunits are associated with collagen-containing subunits through disulfide bonds.
- The homomeric species which can be subdivided into soluble and amphiphilic forms. The amphiphilic forms possess a glycopospholipid (GPI) attachment that interacts with the cell membrane.

The globular form of AChE is assembled in the rough endoplasmic reticulum with the assembly of asymmetric forms occurring approx. 90 minutes later in the distal elements of the Golgi apparatus (Massoulié *et al.*, 1993). Once processed, AChE is packaged in coated vesicles and transported to the plasma membrane, where the vesicles fuse and result in either secretion of AChE or insertion of AChE into the membrane (Rotundo, 1990). The molecular diversity of AChE is not only important for cholinergic transmission but also for the non-cholinergic functions (see later).

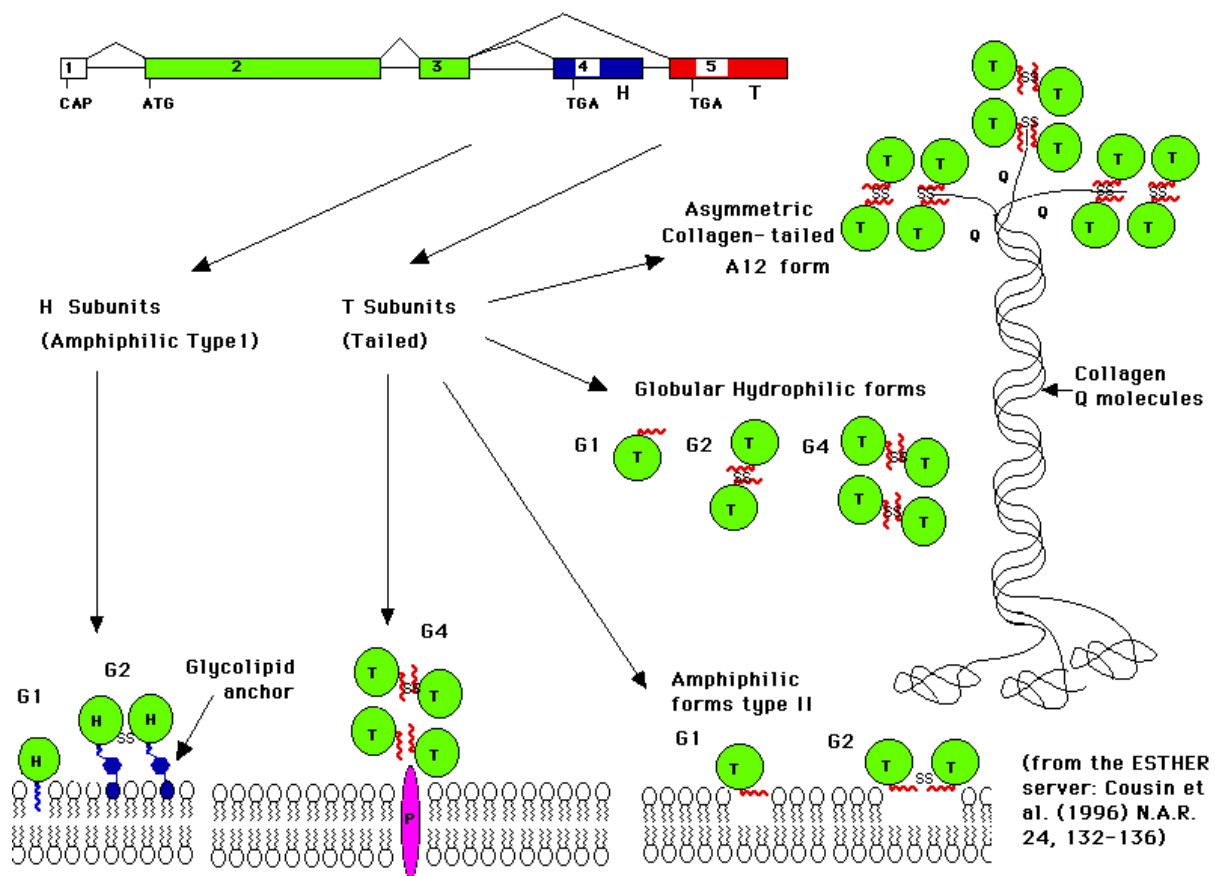


Figure 2. The major oligomeric forms of AChE. The catalytic subunit types are identified by T or H. The molecular form is labeled above. G-globular; A-asymmetric.

1.2. AChE is a multifunctional protein

AChE has been examined extensively in the last century and the idea that AChE has multiple, unrelated biological functions was slowly being accepted. Multiple reviews attempted to give an insight into these functions (Layer and Willbold, 1995; Small *et al.*, 1996; Soreq and Seidman, 2001).

These functions can be classified as below:

1. enzymatic cholinolytic functions (hydrolysis of ACh);
2. enzymatic, non-cholinolytic functions (side activities e.g. arylacylamidase activity);
3. non-enzymatic functions (e.g. adhesion, developmental functions).

Point 1 represents the **classical** function of the enzyme, while 2 and 3 are named **non-classical** or non-cholinergic functions.

1.2.1. The classical cholinergic side of AChE

The effective concentration of neurotransmitter ACh at the neuromuscular endplate and in the central nervous system will be predominantly regulated by the enzyme AChE, which is localized externally on neurites and growth cones, and also can be released into the developing synaptic cleft (see also 1.1.).

1.2.2. Non-classical functions of AChE

The following experimental evidence supported the concept of non-classical functions of AChE:

a. In 1956 Hebb demonstrated that the distribution of choline acetyl-transferase (ChAT) was closely correlated with the distribution of ACh (Hebb, 1956). It might be expected that AChE would similarly localize with ACh, however, a detailed study of the mismatch of AChE- and ChAT- positive structures in the brain indicates that AChE may not only be functioning to hydrolyze ACh (Eckenstein and Sofroniew, 1983). This was the first line of evidence that indicated that AChE may have non-classical functions.

b. High levels of AChE can be detected in the embryonic nervous system well before the period of synaptogenesis (Layer 1983, 1991; Layer *et al.*, 1988a; Robertson *et al.*,

1989). The embryonic expression does not necessarily lead to the expression of the enzyme in the same adult tissues.

c. In paragraph 1.1. the existence of the multiple molecular forms was discussed. The existence of the multiple isoforms of AChE remains unclear if the enzyme has only a single function.

d. Crystallography and sequence analysis have identified a group of related enzymes and non-catalytic proteins. Some of these are transmembrane proteins with cytoplasmic domains and extracellular AChE-homologous domains that share the unique topography of AChE and its strong electric field. On the basis of their structures, all of these are classified as α/β -fold proteins; on the basis of their electric fields they are classified as electrotactins (Botti *et al.*, 1998).

e. AChE has been shown to have direct actions on several types of cells, actions unrelated to a neurotransmission role, e.g. modulation of the neuronal sensitivity (Greenfield *et al.*, 1984; Greenfield, 1991).

Some of the non-classical functions are based on the correlations, other activities have been confirmed and several research groups have established their molecular foundation. The most established ones are discussed below.

AChE regulates neurite outgrowth. The expression of AChE precedes the outgrowth of processes in cultured spheroids (Layer *et al.*, 1988b; Weikert *et al.*, 1990) and in non-cholinergic neurons in the rat thalamus (Kristt, 1989). AChE inhibitors changed neurite growth from chick nerve cells in culture, independent of its enzymatic activity (Layer *et al.*, 1993). Active-site inhibitors failed to attenuate this effect, but an inhibitor of the peripheral site did block neuritogenesis (Layer *et al.*, 1993; Small *et al.*, 1995). Transfection with AChE showed neuritogenic activity of the enzyme in the neuroblastoma cells (Koenigsberger *et al.*, 1997), in phaeochromocytoma (PC12) cells (Grifman *et al.*, 1998) and in primary dorsal root ganglion neurons (Bigbee *et al.*, 2000).

Synaptogenesis. AChE is transiently expressed by thalamocortical axons just prior to synaptogenesis (Robertson, 1987). Increased levels of AChE are detected in maturing neurons in chick spinal cord and in their target myotomes prior to the arrival of axons (Layer *et al.*, 1988a). Thus, axonal pathfinding may be regulated by AChE expression. In the *Xenopus* brain, AChE stimulates the development of axon tracts (Anderson and Key, 1999). However, a direct synaptogenic activity was shown for neuroligin, a non-catalytic transmembrane protein that has an AChE homologous extracellular domain (Scheiffele *et al.*, 2000). When expressed in HEK293 cells, neuroligin induced synaptic vesicle clustering and presynaptic differentiation in adjacent axons. The effect was prevented by the addition

of the extracellular domain of neurexin-1 β , a binding partner for neuroligin (Ichtchenko *et al.*, 1996), which could presumably bind AChE too. This evidence supports the idea that the synaptogenetic role of AChE is probably related to structural properties of the molecule, and not the catalytic function.

Cell adhesion. A cell adhesion function of AChE might provide a mechanism for developmental roles of this protein. There are three major groups of cell adhesion molecules localized on the axonal surface: the cadherins, the integrins, and the members of the immunoglobulin family. Like AChE, these molecules are expressed by the nerve cells prior to synaptogenesis and are responsible for proper outgrowth of the neurites. The adhesive properties of the AChE domain were studied using *Drosophila* cells. Chimeras in which the AChE homologous domain of neurotactin was replaced with the *Drosophila*- or Torpedo-AChE were generated. These chimeras retained the adhesive properties, whereas AChE alone showed no cell adhesion property (Darboux *et al.*, 1996). However, AChE lacks a transmembrane domain and thus cannot send signals into the cell. It was therefore proposed that soluble AChE might compete with the structural homologues (e.g. Neuroligin-1) and in this way send signals into the cell (Grifman *et al.*, 1998). Bigbee and co-workers could provide a direct evidence for an adhesive function of AChE using two different cell (neuroblastoma)-substratum adhesion assays (Sharma *et al.*, 2001). It has been postulated that the adhesion site on the AChE molecule might comprise regions around the peripheral anionic site (Johnson and Moore, 2000). Moreover, recent discoveries showed that the readthrough form of AChE, which accumulates in the neurons under stress conditions, interacts intraneuronally with the RACK1 and PCK β II (Birikh *et al.*, 2003).

Novel functions in physiology – activation of dopamine neurons. AChE is secreted in various areas of the brain including hypothalamus, striatum, hippocampus, cerebellum and the substantia nigra (review in Layer and Willbold, 1995). The cerebellar cortex is a region with low cholinergic transmission; therefore here one has to search for non-classical roles of AChE. The enzyme is released from dendrites of dopaminergic neurons in mammalian substantia nigra (Llinas and Greenfield, 1987). Purified AChE enhanced dopamine release from midbrain dopamine neuron. This prompted the Greenfield group to hypothesize that AChE acts as a neuroactive drug modulating catecholaminergic systems (Greenfield, 1984 a, b).

Amyloid fibre assembly. AChE promotes amyloid fibre assembly (Inestrosa *et al.*, 1996). This activity was blocked by peripheral-site inhibitors (propidium), but not by the active site inhibitor edrophonium, identifying this activity as a non-classical function.

1.3. Binding partners for AChE – an overview

The focus of this thesis was on non-classical, adhesive functions of the enzyme. The actions of AChE on differentiation and proliferation summarized above have been demonstrated in so many experimental systems and laboratories that they now must be considered firmly established functions of the protein. The adhesive functions of AChE involve additional proteins, since the enzyme does not function as a homologous cell adhesion protein.

AChE as a heterophilic cell adhesion molecule. The core domain of AChE is homologous with non-catalytic proteins that have well-defined cell-cell adhesion roles: thyroglobulin and neuroligins in mammals, and gliotactin, glutactin and neurotactins in *Drosophila*. We first proposed that AChE has the same or similar binding partners as those demonstrated for its homologues.

Neurexin-1 β . Neuroligins are transmembrane proteins expressed in the CNS and localized post-synaptically (Song *et al.*, 1999). The neuroligins form a family of cell adhesion proteins encoded by at least three genes. Their extracellular domain possesses ~ 35% homology with AChE. All three known neuroligins bind neurexin-1 β , a pre-synaptic transmembrane protein with a large extracellular domain (Ichtchenko *et al.*, 1996). Neurexin-1 β is a member of a large family of neuronal proteins, composed of at least three genes (neurexins 1 through 3) driven by two promoters (α and β), resulting in the expression of at least six neurexin forms. Alternative mRNA splicing confers additional complexity to the possible gene products (Ichtchenko *et al.*, 1996). The existence of thousands of neurexin isoforms makes them good candidates for cell-cell adhesion molecules. Intracellularly, the neurexins bind to CASK (CaM kinase), PSD95 and synaptotagmin (Missler and Südhof, 1998). The neurexin-1 β & neuroligin-1 couple was shown to promote synaptogenesis (Scheiffele *et al.*, 2000) and mediate recognition processes between neurons by forming heterologous cell contacts at synaptic connections.

Overexpressing human AChE in mice suppressed neurexin-1 β production in embryonic mouse motoneurons (Andres *et al.*, 1997). We therefore considered the possibility that AChE might compete with neuroligin-1 for binding to proteins of the neurexin family and that there is a cross-talk between AChE and neurexin during development. To provide a foundation for this hypothesis first the expression pattern of neurexin-1 β in the retina was examined and compared with AChE.

The developing retina was used for the neurexin-1 β expression studies. The first reason was related to the fact that in the retina only few amacrine cells are cholinergically innervated. On the other hand, AChE is expressed very early in post-mitotic neurons and a

large population of cells expresses AChE during and after the development. This speaks for a predominantly non-classical function of the enzyme in the retina. The second reason was the retina's highly organized laminar structure of alternating cell bodies (nuclear layers) and synaptic connections (plexiform) layers.

Laminin-1. Neurexin-1 β shows three-dimensional similarity to laminins and some lectins in spite of low (10-15%) sequence homology (see Figure 3). Laminin-1 is a part of the basement membrane and thus ideally placed as a potential ligand for AChE in the developing nervous system.

The characterization of laminins started in 1979 with the observation that the Engelbreth-Holm-Swarm (EHS) tumor presents a non-collagenic component in the extracellular matrix. This component was a glycoprotein, named laminin (Timpl *et al.*, 1979). Laminin is the major non-collagenous component of basement membranes and is able to polymerize into a network that is cross-linked with type IV collagen by the single-chain protein entactin/nidogen (Yurchenco and O'Rear, 1994).

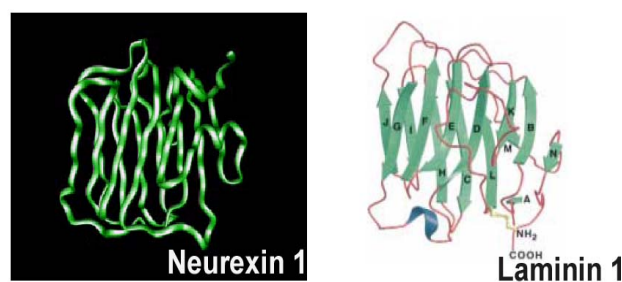


Figure 3. Comparison of the neurexin-1 extracellular domain and laminin-1. (Hohenester *et al.*, 1999)

The laminin molecule is a heterotrimer of three non-identical chains, called α -, β - and γ -chains, according to the new nomenclature (Burgeson *et al.*, 1994). The three chains are members of a multigene family, and so far 5 α (α 1- α 5), 3 β (β 1- β 3) and 3 γ -chains (γ 1- γ 3) have been identified in vertebrates. All laminin chains are multidomain glycoproteins (Figure 4) that contain a 570–600-amino acid α -helical region that forms a triple-stranded coiled-coil with the other two chains (Engvall and Wewer, 1996). The N-terminal regions of the chains are heterogeneous, consisting of alternating globular and cysteine-rich domains of varying size and number. The globular domains are known to be the sites of interaction with receptors and other ligands (Belkin und Stepp, 2000; Andac *et al.*, 1999; Nielsen *et al.*, 2000; Talts *et al.*, 1999). The α -chains contain an additional C-terminal globular domain of approximately 100 kDa (see Figure 4).

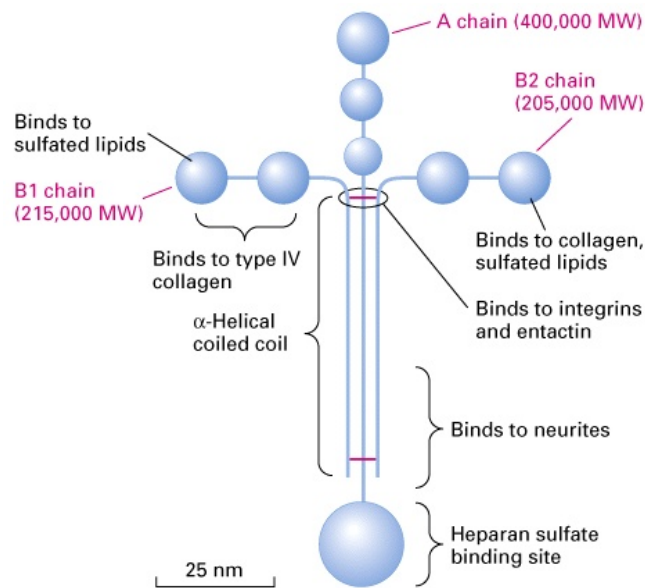


Figure 4. Laminin structure. From the Molecular Cell Biology, 4th edition by Harvey Lodish, Arnold Berk, S. Lawrence Zipursky, Paul Matsudaira, David Baltimore, and James Darnell.

Laminin has many and varied functions that are mediated by binding to various components of the basement membrane (Aumailley and Krieg, 1996; Aumailley and Rousselle, 1999; Colognato *et al.*, 2000). As a cell attachment factor it promotes neurite outgrowth and influences neuron migration, growth, morphology, and adhesion, all functions important in tissue repair. A number of laminin-binding cellular proteins have been characterized, including a variety of cell surface integrins that mediate the interactions of cells with laminin (DiPersio *et al.*, 1997; Belkin and Stepp, 2000). It was also shown that biotinylated human AChE binds to mouse laminin-1 and collagen IV by an electrostatic mechanism (Johnson and Moore, 2003). This *in vitro* study together with the fact that laminin-1 is like AChE very early expressed during embryonic development (Dziadek and Timpl, 1985) provide a basis for interaction studies using other approaches (e.g. yeast two-hybrid, co-immunoprecipitation, surface plasmon resonance). However, more experimental data are necessary and a part of this thesis presents experiments that support the hypothesis and bring more insight into the nature of this interaction. In fact, interaction between AChE and laminin-1 could well be one of the missing links between AChE and other neuronal cell adhesion molecules like integrins. Laminins are known to interact with integrins, a class of receptors coupling proteins from the extracellular matrix with the cytoskeleton, thus linking cell-cell adhesion with cell signaling.

RACK1 and PKC β II. Another important point concerning AChE ligands is the fact that the enzyme is localized extracellularly, soluble or bound to the cell membranes. Therefore, one has to look for extracellular binding partners. However, the normally rare AChE splice variant AChE read-through (AChE-R) is overproduced under stress conditions

in mice (Meshorer *et al.*, 2002), and reduces the stress-induced ACh levels at extracellular sites (Kaufer *et al.*, 1998). Moreover, AChE-R also accumulates in neuronal cell bodies where it interacts with the scaffolding protein RACK1 and through it, with its target, protein kinase C- β II (PKC β II) (Birikh *et al.*, 2003). RACK1 belongs to the family of tryptophan/aspartate proteins, which can simultaneously bind different proteins, e.g. β -integrin, src-kinase or β -adrenergic receptor.

1.4. The Yeast Two-Hybrid System – a genetic approach used to find AChE ligands

A yeast two-hybrid screen was chosen to study possible AChE protein – protein interactions. The yeast two-hybrid system was introduced to the scientific community by Fields and Song in 1989. Most yeast genes are preceded by cis elements called **upstream activating sequences (UASs)**, which are recognized by transcription activators. Eukaryotic transcription activator proteins are composed of at least two domains that can operate independently. The DNA binding domain (BD) binds to specific DNA sequences and the transcription activation domain (AD) initiates transcription by contacting proteins associated with the promoter. Two-hybrid systems make use of the fact that these two domains can be expressed separately, and then forced together non-covalently to form a fully functional transcription activator.

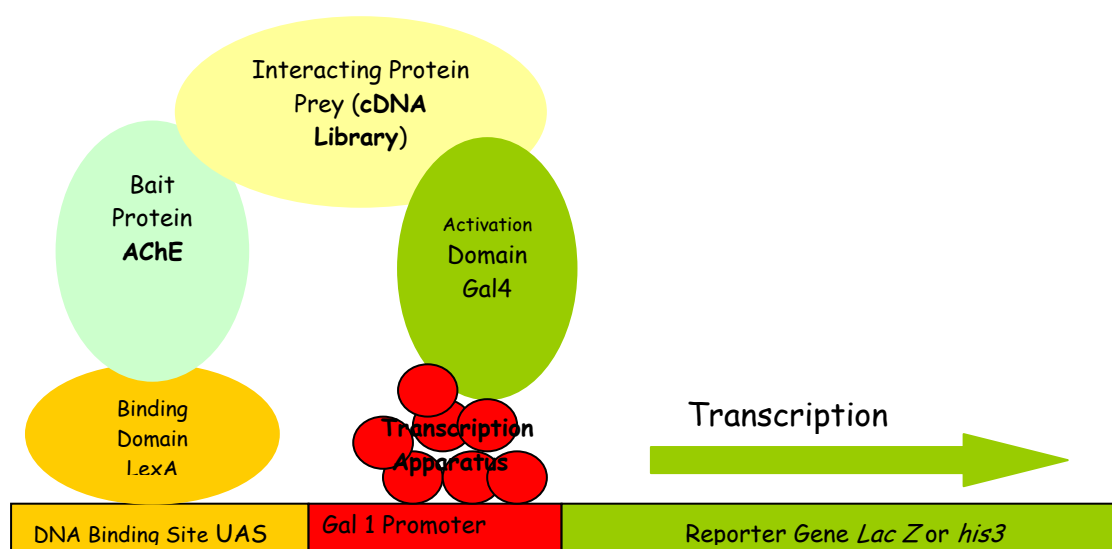


Figure 5. The principle of the yeast two-hybrid system. The interaction between AChE and a library protein reconstitutes the GAL4-transcription activator, which leads to the expression of the reporter gene.

If the binding domain is fused to 'AChE' to create a 'BD-AChE' hybrid, and the activation domain is fused to protein 'X' from a cDNA library to create an 'AD-X' hybrid, then an interaction between AChE and X from the library creates a functional activator which can stimulate transcription of an easily detectable reporter gene. The BD-AChE hybrid is referred to as the 'bait' and the AD-X hybrid is called the 'prey'. In this case, the binding domain of LexA binds to AD of GAL4 to activate transcription of an *E. coli* lacZ reporter gene that is integrated into the yeast genome.

The yeast two-hybrid system was used because it has some clear advantages over classical biochemical and genetic approaches. First of all it is an *in vivo* technique that uses the yeast host cell as a system. This yeast system brings the higher eukaryotic reality closer than most *in vitro* approaches or techniques based on bacterial expression. Appealing features of this system are the minimal requirements to initiate a screening. Only the cDNA, full-length or even partial of the gene of interest is needed, in contrast to sometimes high quantities of purified proteins or good quality antibodies needed in classical biochemical approaches. One of the most appealing features of the yeast two-hybrid system is that the identification of an interacting protein implies that at the same time the corresponding gene is cloned.

1.5. Aims and objectives of the study

The non-classical functions of AChE are intriguing phenomena with important implications aside from the hydrolysis of ACh. The mechanism(s) by which AChE signals its morphogenic functions, however, is a subject of speculation. This gave us the opportunity for planning of new research directions. We hypothesized that AChE is able to send signals into the cell by binding to another protein, that should have the following requisites: be expressed in the same tissues and at the same developmental stages as AChE, have both an extracellular and an intracellular domain connected through one or more transmembrane domains, and possibly be able to initiate an intracellular signaling cascade. Following this hypothesis, we searched for AChE ligands in the central nervous system.

Three approaches have been chosen for this. We first attempted to show that AChE binds to neurexin-1 β , a ligand for a highly AChE homologous protein – neuroligin-1. This binding would explain the synaptogenesis related functions of AChE. Localization studies for neurexin-1 β were also an objective, since there is little known about the expression patterns of this molecule. A second approach was to use surface plasmon resonance and mass spectrometry for finding an AChE unknown partner. Thirdly, a genetic approach (yeast two-hybrid system) was favored to the plasmon resonance and used to screen for unknown

AChE binding partners, considering that yeast systems are generally intensively optimized. The potential AChE binding partners revealed by screening would bring answers to some old questions and at the same time open new questions for the field of cholinesterase research.

2. Materials and methods

2.1. Materials

2.1.1. Instruments

Autoclave	Webeco, Bad Schwartau
Balances	Sartorius, Göttingen
Cell culture hood	Heraeus, Hanau
Centrifuges	Sigma, J2-21, Beckman, USA Eppendorfcentrifuge A14, Jouan
Cryostat	Microm, Walldorf
Electrophoresis chamber (for DNA)	Peq-lab, Erlangen
Electrophoresis chamber (for proteins)	Bio-Rad, München
Electrophoresis power supply	Bio-Rad, Consort E321, München, Biometra Powerpack P25
Electroporator	Bio-Rad, Hercules, California, USA
Freezers	Queue Systems, USA
Heat block	Janke&Kunkel
Incubators, for bacterial culture	Heraeus, Hanau
Incubator, for COS, ES cell culture	Nuaire, Sarstedt
Incubator shaker	New Brunswick Scientific, Edison, USA; WTC
Magnetic stirring plates	Itec, Staufen
Microwave oven	Bosch, Stuttgart
Multipipette	Brand
Microscopes	Axiophot light microscope with fluorescence, Carl Zeiss, Jena Olympus SC 35
pH meter	WTW Wissenschaftlich Technische Werkstätten, Weilheim

Pipettes	Eppendorf, Hamburg
Pipetting device	Hirschmann, Eberstadt
Refrigerators	Privileg, Liebherr, Biberach an der Riß
Rotators	Heidolph Reax 3
Shakers	GFL, Burgwedel
SLT-ELISA reader	Spectra, Crailsheim
Sonicator	Bandelin Electronic, Berlin
Spectrophotometer	Perkin Elmer, Langen
Sterilization oven	Memmert, Schwabach
Thermocycler	Biometra, Göttingen
Ultracentrifuge	Beckman, Krefeld
UV documentation, N-90E	Bender, Wiesloch
Vacuum concentrator, Speedvac	Bachofer, Reutlingen
Vortex	Heidolph, GFL, Burgwedel
Waterbaths	Memmert, Schwabach

2.1.2. General chemicals

Chemicals were of highest purity (for molecular biology or pro analysi).

Agarose	Peqlab, Erlangen
Alkaline phosphatase	Roche, Mannheim
Ampicilline, sodium salt	Sigma, Deisenhofen
APS	Roth, Karlsruhe
ATC	Serva, Heidelberg
ATP	Sigma, Deisenhofen
Bromophenol blue	VWR, Darmstadt
BSA	Sigma, Deisenhofen
CaCl ₂	VWR, Darmstadt
DAB	Sigma, Deisenhofen
Dianisidine	Fulka, Bucsh
Diethanolamine	Sigma, Deisenhofen

DAPI	Boehringer, Mannheim
DMF	VWR, Darmstadt
DMSO	Sigma, Deisenhofen
DNA standard	Roth, Karlsruhe
DTNB(5,5'-Dithio-bis-2-nitro-benzoacid)	VWR, Darmstadt
EDTA·Na ₂ ·2H ₂ O	Sigma, Deisenhofen
Ethanol	Roth, Karlsruhe
Eukobrom Tetenal developer	Hirrlinger, Stuttgart
Ethidium bromide	Roth, Karlsruhe
Formaline 37%	VWR, Darmstadt
Formamide	Sigma Deisenhofen
Gelatin	Sigma, Deisenhofen
Glucose	VWR, Darmstadt
Glycerol	VWR, Darmstadt
H ₂ O ₂	VWR, Darmstadt
HCl	VWR, Darmstadt
HEPES, sodium salt	VWR, Darmstadt
Hexanucleotide mix	Roth, Karlsruhe
Iso-OMPA	Sigma, Deisenhofen
Isopropanol	VWR, Darmstadt
Kanamycine sulfate	Fluka, München
KCl	VWR, Darmstadt
Methanol	VWR, Darmstadt
Milk powder	Roth, Karlsruhe
β-Mercaptoethanol	VWR, Darmstadt
MOPS (3-(N-Morpholino)propanesulfonic acid)	Roth, Karlsruhe
NaCl	VWR, Darmstadt
NaCH ₃ COO	VWR, Darmstadt
Na ₂ HPO ₄	VWR, Darmstadt
PEG 3500	Sigma, Deisenhofen

Phenol	VWR, Darmstadt
Phenol/Chloroform/Isoamyl alcohol	Roth, Karlsruhe
Ponceau S	Sigma, Taufkirchen
Protease inhibitor cocktail	Sigma, Taufkirchen
Protein standards	Sigma, Taufkirchen
Restriction endonucleases, buffers	New England Biolabs,
Salmon sperm DNA	Sigma, Deisenhofen
SDS	VWR, Darmstadt
Sucrose	VWR, Darmstadt
Taq DNA polymerase	Roche, Mannheim
T4 DNA ligase, buffer	Invitrogen, Karlsruhe
TEMED	Roth, Karlsruhe
Thiamine	Fluka, München
Tissue freezing medium	Miles Scientific, USA
Tris	Sigma, Deisenhofen
Tri-sodium citrate·2H ₂ O	VWR, Darmstadt
TritonX100	Sigma, Deisenhofen
Tryptone-peptone	Difco, UK
X-gal	Fluka, München

2.1.3. Chemicals for cell culture

DMEM powder	Gibco, Eggenstein
PBS	Gibco, Eggenstein
FCS	Gibco, Eggenstein
G418 sulfate	Sigma, Deisenhofen
L-Glutamine	Seromed, Berlin
Penicillin/Streptomycin	Cambrex, USA
Trypsin/EDTA	Gibco, Eggenstein

2.1.4. Kits

Plasmid Maxi Kit	Qiagen, Hilden
Nucleospin Extract	Machery-Nagel, Düren
Wizard Miniprep Kit	Promega, Mannheim
Immunoprecipitation starter kit	Amersham, Heidelberg
ECL (enhanced chemiluminescence) Western Blotting Analysis System	Amersham, Heidelberg
Silver Stain Plus	Bio-Rad, München

2.1.5. Consumables

Blotting paper	Schleicher & Schuell, Dassel; Whatman, Maidstone, UK
Cell culture plastic equipment	Greiner, Frickenhausen
Concentrators	Millipore, Bedford, Massachusetts, USA
Electroporation cuvettes	Bio-Rad, Hercules, California, USA
Falcon tubes (14 ml)	Greiner, Frickenhausen
Films	Röntgen films, Sigma, Deisenhofen Polaroid Films, Amersham
Filters	Schleicher & Schuell, Dassel
Glass coverslips	Menzel-Gläser, Braunschweig
Glass slides	Menzel-Gläser, Braunschweig
Hybond-N nylon membranes	Amersham Pharmacia Biotech, Heidelberg
Microcentrifuge tubes	Greiner, Frickenhausen
Nitrocellulose membranes	Whatman Biometra, Göttingen
Parafilm	Neolab, Heidelberg
Pasteur pipettes	Volac, UK
Pipettes (1 ml, 5 ml, 10 ml, 25 ml)	VWR, Darmstadt
Pipette tips	AHN Biotechnologie, Nordhausen
Plastic tubes for bacterial centrifugations	AHN Biotechnologie, Nordhausen

Plastic tubes, sterile (15 ml, 50 ml)	Greiner, Frickenhausen
Plates, for bacterial culture	Greiner, Frickenhausen
PCR tubes	Sarstedt, Nümbrecht
Safe-lock microcentrifuge tubes	Eppendorf, Hamburg
SDS-PAGE gel loading syringe	Hamilton, Switzerland
Spectrophotometry cuvettes	

2.1.6. Enzymes

Pfu DNA polymerase	Promega, Mannheim
Restriction endonucleases	New England Biolabs, USA
Ribonuclease	Roche, Mannheim
Taq DNA polymerase	Eppendorf, Hamburg
T4 DNA-ligase	Invitrogen, Karlsruhe

2.1.7. Media for bacterial culture

LB agar

32 g of LB agar powder were dissolved in 1 L dH₂O and autoclaved. Complete dissolution was achieved only during autoclaving. The solution was cooled to ~ 50°C and antibiotics were added optionally for antibiotic-resistance selection. The solution was poured onto 85 mm or 150 mm plates (~ 40 ml or ~ 100 ml per plate, respectively), and the plates were shortly flamed on a Bunsen burner, to break any air bubbles. The plates were let at RT o/n to allow LB agar to solidify and dry, and kept inverted at 4°C.

Composition: 1% peptone, 0.5% yeast extract, 0.5% NaCl, 1.2% agar.

LB medium

20 g of LB broth base were dissolved in 1 L dH₂O, autoclaved and stored at RT. For antibiotic resistance selection, the antibiotics were added before use.

Composition: 1% peptone, 0.5% yeast extract, 0.5% NaCl.

SOC medium

High quality medium, used for special applications. Obtained ready to use.

Composition: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose.

M9 synthetic minimal medium

Per liter:

5XM9 salts	200 ml
1M MgSO ₄	2 ml
0.1 M CaCl ₂	2 ml
20% Glucose	20 ml
50 mg/ml Ampicilline	1 ml
1 M Thiamine	1 ml

5XM9 salts is made by dissolving the following salts in de-ionized H₂O to a final volume of one liter:

Na ₂ HPO ₄ 7H ₂ O	64g
KH ₂ PO ₄	15g
NaCl	2.5g
NH ₄ Cl	5g

Antibiotic concentrations:

Ampicilline 50 µg/ml, stored as 1000x stock in dH₂O, at -20°C.

Kanamycine 30 µg/ml, stored as 1000x stock in dH₂O, at -20°C.

2.1.8. Media for yeast culture (Gietz, 1992)

YPD-Medium	20 g/L Peptone 10 g/L Yeast extract 2% (w/v) Glucose 30 mg/L Adenine hemisulfate 18 g/L Agar (YPD-Agar) pH 5.8
DO-Medium	6.7 g/L yeast nitrogen base without amino acids 2% Glucose 4 g/L Synthetic Drop out mix (10X) 18 g/L Agar (for SD-Agar) pH 5.6

Synthetic Complete drop-out Medium Mix (SC drop-out) 10X	2.0 g Adenine hemisulfate* 2.0 g Arginine HCl 2.0 g Histidine HCl* 2.0 g Isoleucine 2.0 g Leucine* 2.0 g Lysine HCl 2.0 g Methionine 3.0 g Phenylalanine 6.0 g Homoserine 3.0 g Tryptophan* 2.0 g Tyrosine 1.2 g Uracil* 9.0 g Valine
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The appropriate amino acid(s) (marked with *) were omitted from the SC drop-out in order to prepare DO-His, DO-Leu, DO-Trp, DO- Trp/Leu, and DO-Trp/Leu/His. All the amino acids were purchased from VWR, Darmstadt.

2.1.9. Media for cell culture

Dulbecco's modified Eagle DMEM (Gibco), containing:

10% fetal calf serum, FCS

1% Glutamine

0.1% Penicillin/ Streptomycin.

DMEM-minimal medium (used for transfections)

2%FCS

1% Glutamine

0.1% Penicillin/ Streptomycin.

2.1.10. Bacterial strains

Escherichia coli XL1-Blue (Bullock *et al.*, 1987).

Escherichia coli DH5 α (Hanahan, 1983).

Escherichia coli HB101 (Bolivar und Beckmann, 1979).

2.1.11. Yeast strains

The yeast two-hybrid screen was carried out using reporter strain L40 (Vojtek *et al.*, 1993). Genotype of Strain **L40**:

MATa *his3-delta200 trp1-901 leu2-3,112 ade2 lys2-801am* LYS2::(*lexAop*)4-HIS3 URA3::(*lexAop*)8-lacZ GAL4.

2.1.12. Eukaryotic cell lines

HEK 293 - human embryonic kidney cells (used for transfections and expression of AChE). American Type Culture Collection, Manassas, Virginia, USA.

2.1.13. Yeast two-hybrid cDNA library

Mouse brain MATCHMAKER cDNA library, cloning vector pACT2, (Clontech, Heidelberg, Germany).

2.1.14. cDNA clones

Acetylcholinesterase (AChE) cDNA clone (exons 2, 3, 4, and partially 6)	kindly provided by Prof P. Taylor , UCSD, USA, (Marchot <i>et al.</i> , 1996) cloned in pCDNA3 (Invitrogen)
Neurexin I chicken in pBluescript	kindly provided by Uwe Ernsberger, University of Heidelberg (Patzke and Ernsberger, 2000)
Neurofilament M chicken	generous gift of Uwe Ernsberger, University of Heidelberg (Stanke <i>et al.</i> , 1999)
pLamin cDNA clone (aa 67-230) in <i>pAD</i> -Gal4	generous gift of Prof. Holstein, TU Darmstadt
pCMS-EGFP	Clontech, Heidelberg
p53, in <i>pBD</i> -p53	generous gift of Prof. Holstein, TU Darmstadt
RalAV23, cloned in pBTM116	kindly provided by Jaques Camonis, PhD, Institut Pasteur, Paris, France (Jullien-Flores <i>et al.</i> , 1995)
RLIP (RRall interacting protein) in pGADGH	kindly provided by Jaques Camonis, PhD, Institut Pasteur, Paris, France (Jullien-Flores <i>et al.</i> , 1995)

Synaptotagmin I chicken in pGEM7	kindly provided by Uwe Ernsberger, University of Heidelberg (Campagna <i>et al.</i> , 1997)
Tyrosine hydroxylase chicken in pBluescript	kindly provided by Uwe Ernsberger, University of Heidelberg (Ernsberger <i>et al.</i> , 1995)

2.1.15. Vectors and plasmids

pVJL 10, pVJL 11, pBTM 116, the yeast two-hybrid vectors containing the LexA binding domain, were a generous gift of Jaques Camonis, Institut Pasteur, Paris, France.

pGEX-2T kindly provided by Prof Pfeifer, Institute for Microbiology, TU Darmstadt.

pBluescript, Promega.

2.1.16. Primary antibodies

Goat anti AChE mouse, polyclonal, 200 µg/ml	Santa Cruz Technologies, USA
Mouse anti-AChE chicken, monoclonal, clone 3D10, 2mg/ml	Tsim <i>et al.</i> , 1988
Rabbit anti-Laminin mammalian species, polyclonal, 1:100	Sigma, Deisenhofen
Anti DIG Fab fragments	Roche, Mannheim
Rat anti-Laminin beta1 mouse, monoclonal	Chemicon International, Temecula, California, USA

2.1.17. Secondary antibodies

Donkey anti rabbit, DFAT conjugated	Dianova, Hamburg
Donkey anti rat, biotin conjugated	Dianova, Hamburg
Goat anti mouse, Cy3 conjugated	Dianova, Hamburg
Goat anti rabbit, POX conjugated	Sigma, Deisenhofen
Goat anti rat, POX conjugated	Sigma, Deisenhofen

Rabbit anti goat, POX conjugated	Sigma, Deisenhofen
Rabbit anti goat, Cy3 conjugated	Dianova, Hamburg
Rabbit anti mouse, Cy3 conjugated	Dianova, Hamburg

2.1.18. Synthetic oligonucleotides

Synthetic oligonucleotides were used as PCR primers, as primers for sequencing or for DNA. They were synthesized by Carl Roth GmbH, Karlsruhe.

EcoR1 AChE (left)	5' ATGAATTCGGCCGGGAAGACCCGCAGC 3'
Pst1 2060 AChE (right)	5' TGGGACCCCGTAAACCAG 3'
Sph1 1790 AChE (right)	5' CTCTGGAGGACAGCCCTG 3'
AChE 1430 (right)	5' AGCAGGTCTGGGGTATGG 3'
AChE BamH1 insert orientation (left)	5' AAGATCCTCTCCTCCGTGGT 3'
AChE BamH1 insert orientation (right)	5' ATGCCATGAGTGCAGTGGTA 3'
AChE P2 EcoR1 (left)	5' ATGGAATTCGTGGGCATGCACATA 3'
AChE P2 Bam H1 (right)	5' CTCTCCTCCGTGGTGTAGTC 3'
Gal4AD sequencing primer	5' ATACCACTACAATGGAT 3'
pACT2 insert screen (left)	5' GCGTATAACGCGTTTGGAAT 3'
pACT2 insert screen (right)	5' TTGCGGGGTTTTTCAGTATC 3'

2.2. Methods

2.2.1. Nucleic acid analysis

2.2.1.1. Competent cell preparation

In order to achieve high amplification of DNA, plasmid DNA was inserted into *E. coli* (strain XL1-Blue MRF', DH α 5 or strain HB101) by chemical transformation or electroporation. There are two main methods for preparation of competent bacterial cells for transformation, the calcium chloride and the electroporation method.

2.2.1.1.1. Preparation of chemo-competent bacteria

For the calcium chloride method, a glycerol cell culture stock of the respective *E. coli* strain was thawed and added to 50 ml of LB liquid media. This culture then was pre-incubated at 37°C for 1 hour in a water bath without shaking, transferred to an incubator-shaker, and incubated further for 2-3 hours at 37°C and 225 rpm. The cells were pelleted by centrifugation at 3000 rpm for 8 minutes at 4°C in a Beckman or 6000 rpm in a RC5-B (DuPont), resuspended in one half volume (20 ml) cold 50 mM calcium chloride solution, and incubated on ice for 20 minutes. After another centrifugation step, the resulting cell pellet was resuspended in one tenth volume (4 ml) of cold, sterile 50 mM calcium chloride to yield the final competent cell suspension. Competent cells were stored at 4°C, for up to several days or glycerol can be added to a final concentration of 17%, so that the cells can be placed at -70°C and stored indefinitely.

2.2.1.1.2. Transformation of chemo-competent bacteria (Hanahan, 1983)

In order to use competent cells for transformation, 100 μ l aliquots were removed from freezer and thawed for a few minutes at RT. Plasmid DNA was added (~10 ng) and incubated with the cells for 15 minutes as in the standard transformation procedure. This time period allows the plasmid DNA to attach to the cell membrane. The bacteria were heat shocked at 42°C for 2 minutes (during which the plasmid DNA enters the cell), cooled briefly, and incubated for 1 hour at 37°C in one ml LB before spreading on selective plates.

2.2.1.1.3. Preparation of electro-competent bacteria

50 ml of LB liquid bacterial growing medium was inoculated with a single colony from an LB agar plate. Bacterial cells were grown at 37°C, o/n, shaking at 225 rpm. 1 L of LB medium was inoculated with 10 ml of the above culture. Cells were grown at 37°C for 3-5 h, until they reached an OD₆₀₀ of ~ 0.6-0.7. Cells were centrifuged at 1500 g, 4°C, for 15 min and resuspended in 1 L of ice-cold 10% glycerol. The centrifugation and resuspension steps were repeated two times with 500 ml and 250 ml of 10% glycerol and cells were finally resuspended in 4 ml of 10% glycerol. Cells were shock-frozen in liquid nitrogen in 40 µl aliquots and stored at -80°C.

2.2.1.1.4. Electro-transformation (electroporation) of bacteria with plasmid DNA

2 µl of plasmid DNA (ligation mixture, diluted 1:4 with TE or yeast plasmid preparation, diluted with TE) were mixed with 40 µl of electro-competent bacteria (which was thawed on ice) and kept on ice for 1-5 min. The mixture was transferred to an ice cold electroporation cuvette and pulsed at 2.5 kV in an *E. coli* pulser (Bio-Rad). In order to allow bacteria to recover, they were transferred to 1 ml SOB medium and incubated at 37°C for 1 hour, shaking at 250 rpm. For mini DNA preparations, bacteria were plated on 85 mm LB agar plates, containing the appropriate antibiotic, and grown at 37°C o/n. For maxi DNA preparation, 4 ml of LB medium, containing the appropriate antibiotic were added to the transformed bacteria and incubation was continued at 37°C for 4 h. 1 ml of the culture was used to inoculate 500 ml of LB medium with the appropriate antibiotic and cells were grown at 37°C o/n.

2.2.1.2. Plasmid DNA preparations

2.2.1.2.1. DNA mini-prep

5 ml (mini) cultures were inoculated with single colonies from LB agar plates and grown o/n. DNA was extracted and purified, using the Wizard Plus Miniprep Kit (Promega), and stored in 10 mM Tris-HCl, pH 8.5 at 4°C. Purification of plasmid DNA with this kit is based on an alkaline lysis procedure, followed by adsorption of plasmid DNA onto a silica-gel membrane. Purified DNA was analyzed with restriction enzymes and, optionally, with DNA sequencing.

For cloning and subcloning a Birnboim (1983) protocol was used. Starting with 2 ml o/n cultures (LB, other *E. coli* broths), 1.5 ml were poured into 1.5 ml microfuge tubes and centrifuged 1 min in a microfuge (bacteria were pelleted). After carefully aspirating off the medium, 150 μ l of Solution I (isotonic solution) were added and the pellet was resuspended by vortexing. 300 μ l of Solution II (lysis solution) were added and mixed completely by inverting the microfuge tube. The cells should lyse and turn somewhat clear and viscous. After ~3 min incubation on ice, addition of 225 μ l Solution III, and mixing by inversion, a white clot of DNA/protein/SDS should form. The mixture was incubated on ice 10-30 min and centrifuged for 15 min at 12000g in a microfuge. The supernatant (~400 μ l) was transferred into a fresh 1.5 ml microfuge tube and the DNA was precipitated with 1 ml 95% ethanol. After centrifugation in a microfuge for ~15 min at max speed, the ethanol was decanted and the pellet dried in speed vac or air dry. The pellet was resuspended in 50 μ l water or TE (the resuspension volume was dependent on copy number of plasmid). The plasmid DNA was now ready for restriction digestion, PCR, subcloning, etc. These DNA preparations contain RNA; therefore in case of complications a standard RNase treatment may be applied. Further cleanup can be accomplished with phenol/chloroform extractions but for routine screening and subcloning this was often not necessary.

Solution I	Solution II	Solution III
5 mM sucrose 10 mM EDTA 25 mM Tris, pH 8.0	0.2 N NaOH 1% (w/v) SDS	3 M sodium acetate, pH 4.8

2.2.1.2.2. DNA maxiprep

For most cloned constructs, a higher and better purified amount of DNA was prepared by the maxiprep procedure. DNA was extracted and purified using the Qiagen Plasmid Maxi Kit, and stored in TE (see below) at -20°C. Purification of plasmid DNA with this kit is based on an alkaline lysis procedure, and the binding of plasmid DNA to an anion-exchange resin. The composition of purified DNA was confirmed with restriction analysis.

TE buffer - 10 mM Tris, 1 mM EDTA·Na₂⊕2H₂O, pH 8.0.

DNA is more stable in slightly alkaline conditions; EDTA stabilizes DNA by forming a complex with Mg⁺⁺, which is required for DNA hydrolysis by DNases.

2.2.1.3. Restriction enzyme analysis of DNA

In order to check the composition of purified nucleic acids, DNA was digested with restriction endonucleases. Digestions were carried out for 1 hour to o/n, in 10 µl reaction volume; in the optimal buffer for each endonuclease (suggested and provided as 10x concentrations by New England Biolabs or Promega). The digestion temperature was 37°C for most endonucleases, although with some enzymes an o/n cut was made at 4°C. In some cases, double enzyme digestions were also carried out. The buffers used were determined by a compatibility table, provided by Promega. In cases where the optimal temperature of the two enzymes was different, the digestion was performed first at the lower temperature (usually 37°C) and then at the higher temperature.

2.2.1.4. Agarose gel electrophoresis

During agarose gel electrophoresis, DNA fragments are separated according to their size, the smaller a fragment is, the faster it migrates. The size of a fragment is estimated by the comparison to the sizes of the bands of a DNA molecular weight marker. Agarose, in a concentration of 0.7-1.5%, was dissolved in 1x TAE in a microwave oven. The solution was cooled to ~ 60°C and ethidium bromide was added, at final concentration 1 µg/ml. The solution was poured into a gel chamber with a well separator and let to solidify at RT. The concentration of the gel reflects the area of DNA size where separation is required to be optimal, large fragments (> 3 kb) separate better in low concentration gels, and small fragments in high concentration gels. DNA samples were mixed with 1/10 volume of 10x loading buffer (see below) and loaded into the wells of the gel. Gels were electrophoresed at 80-120 V for 30 min - 4 h in 1x TAE. The DNA bands, based on their binding to ethidium bromide, were visualized under UV light.

Buffers:

50x TAE: 2 M Tris, 50 mM EDTA·Na₂⊕2H₂O, 4% acetic acid, pH 8.5.

10x loading buffer: 57% glycerol, 100 mM Tris pH 8.0, 10 mM EDTA·Na₂⊕2H₂O, ~ 0.001% bromophenol blue.

2.2.1.5. Purification of DNA from preparative digestions

DNA digestions for cloning purposes (preparative digestions) were carried out o/n, in 30 µl reaction volume. Digested DNA was either run on an agarose gel to separate different fragments or used directly (usually vector digestions). DNA was purified from agarose gels

either using the Nucleo Spin Extraction Kit (Macherey-Nagel), according to manufacturer's protocol, or by glass wool: phenol/chloroform extraction. When the second method was followed, the DNA band was collected from the gel, cut into small pieces with a scalpel and introduced into a 500 µl microfuge tube. The tube has a small hole at the bottom and 1/5th was filled with glass wool to prevent the contamination of the DNA solution with agarose. The 500 µl microfuge tube was inserted into a 1.5 ml microfuge tube and the DNA was collected by centrifugation 10 min at 8000 rpm. The method is based on the property of DNA to be water soluble. The collected solution was further purified by phenol/chloroform extraction.

2.2.1.6. Phenol/chloroform extraction and ethanol precipitation of DNA

Phenol/chloroform is used to purify and concentrate DNA from different preparations. Such extraction is used to inactivate and remove enzymes that are used in one step of a cloning operation. The procedure takes advantage of the fact that protein removal is more efficient when two different organic solvents are used instead of one. Phenol denatures proteins very efficiently, but does not completely inhibit RNase activity, and acts as a solvent for RNA molecules; this is the reason for using mixture of phenol:chlorophorm:isoamylalcohol (25:24:1) (PCI). The DNA solution was transferred to a microfuge tube and an equal amount of PCI was added. The contents were mixed (vortexed) until an emulsion was formed and then centrifuged for 15 s at max speed on a microfuge at RT. The aqueous phase is formed as the upper phase, normally. With a pipette the upper phase was transferred to a new tube and the nucleic acids were recovered by precipitation with 2 volumes 100% ethanol and 1/10 volume of sodium acetate 3M and centrifugation for 30 min at 4°C and 12000 rpm.

2.2.1.7. Ligation

DNA ends of different or the same DNA fragment were ligated by incubation with T4 DNA ligase at 12-15°C or 4°C o/n. Ligation is the formation of a covalent bond between a 5' phosphate and a 3' hydroxyl group. Ligation reaction mixture included 10 - 100 ng of vector DNA and 1-5 times more "insert" DNA, in molecular quantities.

The amount of necessary insert was calculated with the following formula:

$$\text{ng Insert} = \frac{\text{Size of Insert (bp)} \times \text{amount of Vector (10 to 100 ng)}}{\text{Size of Vector (bp)}}$$

The reaction was carried out in 10 µl volume, using 1 µl (4U) of T4 DNA ligase, in 1x ligation buffer (it was provided with the ligase and included the required ATP).

2.2.1.8. Measurement of DNA concentration

DNA concentration was estimated using agarose gel electrophoresis, by comparing the intensity of a small amount of purified DNA with that of 10 µl of 0.1 µg/µl DNA molecular weight marker (Roth). For more accurate quantitations, the OD at 260nm of a specific dilution (usually 1:20) of purified DNA was measured. The concentration of DNA was calculated according to the following formula:

double-stranded DNA: 1.0 OD₂₆₀ unit = 50 µg/ml

or

OD₂₆₀ X dilution factor X DNA factor (50) = µg DNA / µl

2.2.1.9. PCR (polymerase chain reaction) (Saiki *et al.*, 1985)

The Polymerase Chain Reaction enables the synthesis of specific DNA sequences using two oligonucleotides complement to the two DNA strands. The reaction requires a Thermocycler, to control the exact temperature for each of the different steps that characterize the PCR: melting, annealing, and amplification. The amplification reaction is carried out by the heat resistant polymerase named Taq DNA polymerase. This technique provides a high variety of application possibilities, outgoing from different types of templates, as DNA, RNA or cDNA. Thus, the PCR can be used to confirm the presence of a DNA fragment in a plasmid, the expression of certain gene (RT-PCR) or can be used to prepare DNA fragments for cloning. The PCR was used in this work to generate fragments for cloning and to check the presence and orientation of a specific insert in a vector.

Standardized PCR protocol

Reaction mix:

DNA	10-100ng
-----	----------

PCR-buffer1x	
dNTPs	200µM
MgCl ₂	between 1mM and 5mM
Primer left	100pmol
Primer right	100pmol
Taq-polymerase	2.5U/ 50µl reaction volume
H ₂ O	to a final volume of 50µl or 100µl

Cycle parameters:

94°C 2 min 1 cycle

94°C 1 min 30 cycles

60°C 1 min (temperature depends on the T_m on the applied Primers)

72°C X min (time depends on the length of the fragment; 1kb/min)

72°C 5 min 1 cycle

PCR buffer 10x: 500 mM KCl, 200 mM Tris-HCl, pH 8.6, 1% Triton X-100.**2.2.1.10. Colony PCR (yeast)**

In order to check up the expression of different genes on a yeast colony, we employed PCR on yeast colonies, a fast and easy control method for expression studies. A bit of a colony was trashed into a PCR tube, 50 µl H₂O were added, and the mixture was boiled for 10 min and centrifuged 5 min at 12000 rpm at RT. 1 to 5 µl were used for a regular PCR reaction. Reaction mix (see up)

Cycle parameters

95°C 3min	
55°C 1min	2 cycles
72°C 3min	

95°C 3min	
55°C 1min (temperature depends on the T _m of the applied Primers)	30 cycles
72°C 3min	

2.2.1.11. Sequencing of DNA

Sequencing of DNA clones was performed automatically using oligonucleotides labeled with fluorescence (Automated Laser Fluorescence) and using the Applied Biosystems sequencing system (Pharmacia). The Sequencing process was performed by the SeqLab Laboratoires (Göttingen, Germany).

2.2.2. Yeast-Two-Hybrid Screen (Fields and Song, 1989)

Through the Yeast-Two-Hybrid-System is possible to identify protein-protein interactions *in vivo*. The Yeast-Two-Hybrid-System was used to identify *in vivo* interaction partners for AChE. The system is based on the properties of eukaryotic transcription factors that possess two functional independent domains that can be physically separated. In this thesis, the DNA binding domain was fused to the AChE (BD-AChE fusion protein), and the trans-activating domain was fused to the proteins encoded by the cDNA library (AD-translated cDNA fusion proteins). The co-expression of these proteins was carried out in yeast containing reporter genes (such as *Escherichia coli* LacZ and the yeast gene His) that require the activity of the transcriptional activator for expression.

The bait protein AChE was cloned in the pVJL11 vector (gift of J. Camonis) and fused with the LexA binding domain. The mouse cDNA library was cloned in the vector pACT2 (Clontech, Heidelberg) and fused with the Gal4-AD. Both plasmids were transformed into L40 yeast strain, where they were expressed and transported to nucleus. In the case that AChE interacted with one of the library proteins, LexA-DNA-BD comes close to GAL4-AD and the reconstitute the transcription activator. The transcription factor was then able to activate the transcription of reporter genes (*lacZ* and *HIS3*).

2.2.2.1. Construction of the hybrid "Bait"

The complete open reading frame of mouse AChE was cloned in frame in pVJL11 vector as a fusion construct between C-terminus of LexA binding domain and N-terminus of AChE. An AChE-LexA hybrid protein would be expressed by transforming the yeast cells with this construct. The hybrid protein was not activating the GAL4 gene, this being one of the basic conditions that has to be fulfilled

2.2.2.2. Mouse fusion cDNA library

A Clontech Matchmaker mouse brain library was used. The cDNAs were cloned using the *XhoI* / *EcoRI* restriction sites in the pACT2 vector. The size of the cDNAs varied between 0.4 -4.0 kb and the average cDNA size was 2.0 kb. The number of independent clones 3.5×10^6 and the estimated % of colonies with inserts was 95%. However, the library data were tested and the estimated % of colonies with inserts was about 60%. The titer of the library was 0.335×10^9 cfu/ml (colony forming units).

2.2.2.3. Small scale yeast transformation

The first step in setting up a two-hybrid system was to transform the bait plasmid into the yeast strain L40 and to select the yeast transformants on a minimal medium lacking tryptophan. It was absolutely necessary to rule out the possibility of the 'bait' plasmid to trans-activate the reporter gene by itself. In order to test this, a small-scale yeast transformation protocol was used. Competent yeast cells were prepared using the lithium acetate (LiAc) method.

10 ml YPD or DO medium were inoculated with a large L40 colony and shaken o/n at 30°C. The next day, the 10 ml cell culture was diluted in 100 ml YPD and grown for additional 3 to 5 hours at 30°C. The cells were then pelleted at 3500 rpm for 5 min at RT, resuspended in 50 ml 0.1 M LiAc in TE pH 7.5, pelleted again and resuspended in 2 ml of 0.1 M LiAc in TE. After one hour shaking at 30°C the yeast cells were ready for transformations (competent). They could also be kept overnight at 4°C for transformation next day.

200 ng DNA were mixed with 100 µl competent yeast cell suspension and 500 µl 50% PEG, 100 mM LiAc, 1XTE, vortexed and incubated at 30°C for 30 minutes. The cells were heat shocked for 15 to 30 minutes at 42°C, pelleted at full speed for 5 seconds, washed in 1 ml YPD and re-centrifuged and resuspended in 1 ml YPD. 100 µl cells were plated on DO/-Trp medium and incubated at 30°C for 2-3 days.

2.2.2.4. Large scale yeast transformation (library transformation, Michael White)

To perform a two-hybrid screen, two-hybrid plasmids must be introduced by transformation into the yeast reporter strain, either simultaneously or sequentially. The simultaneous co-transformation is preferable to sequential transformation if the expression of 'bait' protein is toxic to the cell. Otherwise the sequential transformation is recommended

because it gives higher transformation efficiency. For the sequential transformation, the library plasmids are introduced into competent yeast cells that have been previously transformed with the AChE bait hybrid plasmid. The most efficient screen for yeast transformants containing interacting proteins is to select transformants for the activation of the *his3* reporter gene. The yeast L40 strain containing the AChE 'bait' is transformed with the pACT2 cDNA library plasmids. A typical two-hybrid screen requires 5×10^6 transformants.

One or two colonies containing the bait plasmid were grown in 25 ml DO/-Trp o/n at 30°C on a shaker. The next day the cells were counted and diluted in 250 ml selective medium DO/-Trp at 5×10^6 cells/ml. After growing the cells for further 3 hours at 30°C to a concentration of 1×10^7 cells/ml, the suspension was centrifuged for 5 min at 3000-5000 rpm, RT, and the pellet was washed in 100 ml sterile water, and re-centrifuged. Another wash step followed, and the pellet was this time re-suspended into 50 ml of 0.1 M LiAc in TE, followed by centrifugation for 5 min at 3000 rpm, and the pellet was resuspended in 10 ml 0.1 M LiAc/TE and finally, incubated on a shaker at 30°C for one hour. The now competent L40 yeast cells were pelleted, and resuspended in 2 ml 0.1 M LiAc/TE. To the cell suspension 60 µg library plasmid DNA and 20 ml 40% PEG 3350 in (LiAc 0.1 M/Tris-HCl pH 7.5 10 mM/ EDTA 1mM) were added. The mixture was well vortexed and incubated at 30°C to allow the plasmids to attach the yeast cell. The L40 cells were heat shocked for 30 min at 42°C to allow the library plasmids to enter the yeast cells. 2 wash steps followed: both in 10 ml YPAD preceded by centrifugation at 3000 rpm, 5 min, RT. In order to increase the transformation efficiency and to allow cell regeneration (*His* selection shouldn't be applied too short after transformation), the yeast cells were resuspended into 250 ml DO/-Trp/-Leu, and grown for about 8 hours at 30°C. The transformants were pelleted and resuspended in 2 ml DO/-Trp/-Leu and aliquots were plated onto 10XØ140mm DO/-Trp/-Leu/-His plates. With this protocol the first positive clones were visible between days 3 and 5. The cells that could grow on these plates were selected for the presence of both pLexA-AChE and pGAL4cDNA library plasmid and the activation of the *his3* reporter gene.

In order to eliminate false-positive clones the activation of the second reporter gene, *LacZ*, was tested by analyzing the β-galactosidase activity (with the filter lift-assay).

2.2.2.5. β-Galactosidase-Filter lift -Assay (Breedon and Nasmyth, 1985, modified by Neiman)

The β-Galactosidase-Filter-Test is a sensitive assay to determine whether yeast colonies on a plate express β-galactosidase activity. The assay could be performed any

time after colonies became visible. The yeast cells prepared as patches were replicated with a replicating cylinder and a velvet on a Whatman paper laid on a plate containing the same medium as the one used to grow the cells. After incubating o/n at 30°C, the filter with the grown yeast cells was sunk into a liquid nitrogen container for 15 s. The stiff filter was laid on another filter soaked with the X-Gal/Z buffer (see below). After 2 to 18 hours incubating the filter at 30°C in dark, colonies expressing LacZ gave a more or less deep blue color. Strong positive colonies turn blue as soon as 30 minutes, weaker positives may take overnight or longer to turn blue.

Z buffer.

- 60 mM Na₂HPO₄
- 40 mM NaH₂PO₄
- 10 mM KCl
- 1mM MgSO₄, pH 7.

Z Buffer/X-Gal solution: 100 ml buffer Z, 0.29 ml 2-mercaptoethanol and 1 ml X-Gal (2% in dimethyl-formamide).

2.2.2.6. Selection of transformants

The colonies His⁺/LacZ⁺ identified by above procedures contained both the AChE and the library hybrid plasmids. To further analyze these cDNA clones, it was necessary to separate the library plasmid from the bait AChE. *E. coli* strain HB101 had a defect in the *leuB* gene, which could be complemented by the *LEU2* gene from yeast. The cDNA library vector pACT2 had the *leu*, but not the pLexA-AChE. Thus, pACT2 library plasmids could be separated from the bait AChE plasmid by transforming HB101 with the plasmid DNA isolated from the His⁺/LacZ⁺ clones. The bacterial transformants growing on minimal M9 medium lacking leucine would harbor only the pACT2 hybrid plasmids. Since the plasmid DNA isolated from yeast contains a lot of genomic DNA, electroporation is recommended to introduce plasmid DNA into bacteria (see 2.2.1.1.4.).

2.2.2.7. Isolation of plasmid DNA from yeast (Hoffman and Winston, 1987)

His⁺/LacZ⁺ colonies were cultivated in 2 ml DO/-Leu o/n at 30°C on an incubation shaker. The yeast cell suspension was then centrifuged for 5 s to pellet the cells. The pellet was resuspended in 100 µl TE, 200 µl Lysis solution, 200 µl phenol:chloroform:isopropanol:25:24:1, and 300 mg glass beads. The mixture was vortexed for 2 min and centrifuged for 5 min at 14000 rpm RT. The supernatant was transferred to a

new centrifuge tube with 0.1 volumes potassium acetate (5M) and 2 volumes of ethanol 100%. The precipitated plasmid DNA was then recovered by centrifugation 5 min at 14000 rpm RT, washed with 70% ethanol and air or vacuum dried. The pellets were resuspended in 20 µl TE and 1-2 µl was used to electroporate bacteria.

Yeast lysis solution:

2% Triton X-100

1% SDS

0.1M NaCl

0.01M Tris (pH 8)

1 mM EDTA.

2.2.2.8. Preparation of yeast protein extracts

10 ml L40 culture at 2×10^7 cells/ml (OD 0.7-0.9) were centrifuged 10 min at 3000 rpm, re-suspended in 1 ml H₂O and transferred into a microfuge tube. The suspension was centrifuged again and the pellet was frozen in liquid nitrogen and re-suspended in 400 µl sample buffer. After some minutes of incubation on ice, 400 µl glass beads were added and vortexed 4 times 30 s on a maximum speed. Between each vortex step the probes were boiled for one min. After 15 min centrifugation on max speed the supernatant (about 100 µl) was transferred into a new centrifuge tube and kept at -20°C.

Sample Buffer:

2% SDS

100 mM DTT

60 mM Tris pH 6.8

0,001% Bromphenol blue

10% Glycerol

Glass beads (Sigma) 0.45 – 0.50 mm diameter, soaked in 1 N HCl o/n, and washed in H₂O.

2.2.2.9. DNA database search

DNA databases were searched in order to identify the protein sequences of the different AChE binding partners or to compare already known sequences. The searches were performed using the internet site of the National Centre for Biotechnology Information, USA (URL: <http://www.ncbi.nlm.nih.gov>). Two types of searches were carried out: GenBank search and Basic Local Alignment Search Tool (BLAST) search. In the GenBank database (National Institutes of Health, Bethesda, Maryland, USA), DNA sequences are annotated

and if a potential protein sequence shows similarity with an already known protein, this is indicated in the name of the submitted sequence. Therefore, for the current study, a search for AChE was performed. BLAST search of a database (Altschul *et al.*, 1990) is carried out using a DNA or protein sequence as a query, and results in the identification of sequences with significant homology to that of the query sequence or of a part of it. A DNA database can be searched also with a protein sequence (translated nucleotide database search), thus enabling identification of similar protein sequences (e.g. of different protein isoforms) that are derived from not significantly similar DNA sequences. The sequences obtained from the searches were analyzed on a PC, using Genedoc software.

2.2.3. Protein analysis

2.2.3.1. The Bradford Assay (Bradford, 1976)

The Bradford protein assay is one of several simple methods commonly used to determine the total protein concentration of a sample. The method is based on the proportional binding of the Coomassie dye to proteins. Within the linear range of the assay (~5-25 µg/ml), the dye-protein binding is also linear. Furthermore, the assay is colorimetric; as the protein concentration increases, the color of the test sample becomes darker. The protein bound Coomassie Brilliant Blue G-250 absorbs at 595 nm (Unbound dye has also another absorbance wavelength, R-250 likewise). The protein concentration of a test sample is determined by comparison to that of a series of protein standards known to reproducibly exhibit a linear absorbance profile in the assay. Although different protein standards can be used, the most widely used protein standard, Bovine Serum Albumin (BSA), was chosen.

Procedure

A BSA standard reference curve was generated from a standard 1 mg/ml BSA stock solution: 100, 80, 60, 40, 20, 10, 0 µg/ml. For the microtiter plate assay 20 µl sample and 200 µl Bradford reagent were mixed for 10 min at RT. OD is measured at 620 nm in a microplate reader. For the standard test, 100 µl of probe were mixed with 1 ml Bradford reagent and the OD was measured at 595 nm.

Bradford reagent

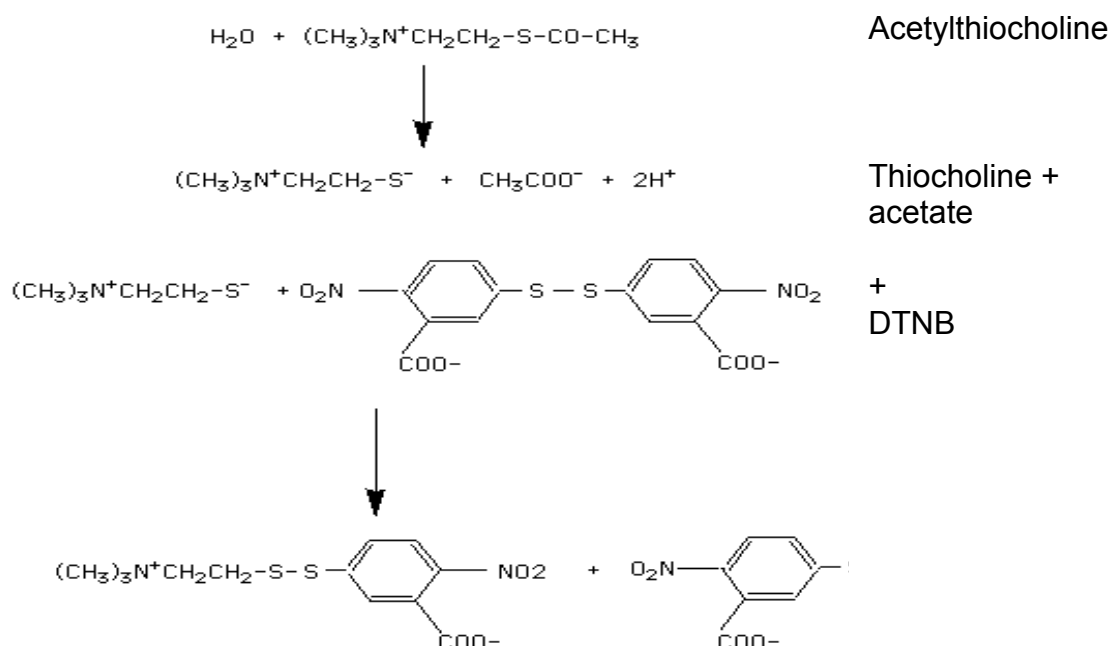
0.01% Coomassie Brilliant Blue G-250

4.7% ethanol

8.5% H₃PO₄

2.2.3.2. Ellman activity assay (1961)

The use of thiocholine esters together with the Ellman reagent allows colorimetric detection of cholinesterases. This type of reaction in which the free sulfhydryl product of the enzymatic hydrolysis reacts with 5, 5'-dithio-bis(2-nitro benzoic acid) (DTNB) is presented below:



The assay measures the increase of OD at 412nm (due to the formation of a yellow product) with the time, in a 3 minutes interval. The following reagents were pipetted in a spectrophotometer cuvette:

2 mM acetylthiocholine iodide (substrate for AChE)

40 mM MOPS

0.25 mM DTNB

10^{-5} M Ethopropazine (inhibitor for BChE)

50µl Probe

to an end volume of 500µl.

The activity was measured and calculated using the KinLab software from Perkin Elmer.

2.2.3.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli, 1970)

By SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated according to their molecular weight. All solutions used are Tris-based buffers, containing

SDS. The function of SDS is to unfold the proteins by binding to their hydrophobic groups and to provide them with a negative charge, irrespective of their isoelectric point. Before subjected to electrophoresis, proteins are mixed with a buffer, apart from SDS, which also contains β -mercaptoethanol, a reducing agent that breaks disulfide bonds and thus separates multisubunit proteins to their monomers. The effect of β -mercaptoethanol is assisted by heating the sample at 100°C.

The electrophoresis gel consists of two parts: a) the stacking gel, a low concentration gel of pH 6.8 which functions to focus the different proteins of the loaded samples and b) the separation gel, a higher concentration gel of pH 8.8 in which proteins are separated. The separation gel was first prepared (see below). The concentration of the gel varied, according to the area of protein size where separation was required to be optimal, low molecular weight proteins separate better in high concentration gels and high molecular weight proteins in low concentration gels. The mixture was loaded into the running cassette and overlaid with H₂O. The stacking gel was prepared (see below), loaded on top of the separation gel and a well separator was inserted. After polymerization, the running cassette was placed into a chamber, which was filled with running buffer (see below). The well separator was removed and wells were washed with running buffer. Protein samples were mixed with an equal volume of 1x sample buffer (see below), heated at 100°C for 3-5 min and loaded into the wells of the gel. A molecular weight standard was also loaded, which enabled the estimation of the molecular weight of the proteins analyzed. Pre-stained standard was used for western blot or Coomassie-blue staining application, respectively. Electrophoresis was carried out at 150 V for 1 h.

Gels were used either for Western blot or for Coomassie-blue staining. The composition of the gel mixtures, as well as of the other solutions used is shown below:

	<i>Separation gel (7.5%)</i>	<i>Stacking gel (4%)</i>
dH ₂ O	4.8 ml	3 ml
Tris buffer	2.5 ml (1.5 M, pH 8.8)	1.25 ml (0.5 M, pH 6.8)
30% Acrylamide 0.8% bis acrylamide	2.5 ml	0.65 ml
APS 10%	100 μ l	100 μ l
SDS 10%	50 μ l	50 μ l
TEMED	5 μ l	5 μ l

Running buffer:

0.25 M Tris, 1.9 M glycine, 1% SDS, pH 8.8

6x Sample buffer:

0.125 M Tris, 4.6% SDS, 20% Glycerol, 5% β -mercaptoethanol, small amount of bromophenol blue, pH 6.8

2.2.3.4. Western blot (Towbin *et al.*, 1979)

Western blot analysis enables high sensitivity detection of a specific protein in a protein mixture, when an antibody against an epitope of a particular protein is available. The various proteins of the mixture are separated in an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane is then subjected to immunodetection.

2.2.3.4.1. Transfer of proteins to a nitrocellulose membrane

After SDS-PAGE, the stacking gel was removed and the separation gel was assembled together with the membrane wetted with transfer buffer. Four sheets of blotting paper were placed on both sides of the assembly. Proteins were transferred electrophoretically at 300 mA for 1-2 h. The membrane was stained with Ponceau-S (see below) for 1-2 min, a reversible stain, to verify protein transfer. Protein bands were visualized after destaining with H₂O. The membrane was further destained with T-PBS (phosphate buffer saline (PBS) with 0.05% Tween 20) for 1-2 min. Solutions used:

Transfer buffer: 0.025 M Tris, 0.19 M glycine, 20% methanol.

Ponceau-S stain: 5% acetic acid, 0.2% Ponceau-S.

2.2.3.4.2. Immunodetection

For the immunodetection of the protein of interest transferred on a nitrocellulose membrane, the membrane was first incubated with a blocking buffer containing a rich source of natural proteins. The reason for this blocking was to allow weak, unspecific binding of the protein source of the buffer to all the proteins transferred onto the membrane. The membrane was then incubated with an antibody against an epitope of the protein of interest (primary antibody) in blocking buffer for 1h at room temperature or o/n at 4°C. The antibody binds to the protein of interest in a competitive fashion with the proteins of the blocking buffer. This results in high specificity of binding and elimination of background. After washing 3x5 min with T-PBS the membrane was incubated with a secondary antibody, also in blocking buffer, which binds to the primary antibody. The secondary antibody is conjugated with horseradish peroxidase (HRP). Incubation with the secondary antibody

(1:10000, unless otherwise stated) was done in blocking buffer at RT for 60 min. After washing 3x15 min with T-PBS the membrane was developed using a chemiluminescence detection kit (Amersham).

Blocking buffer: 5% milk powder in T-PBS

T-PBS: PBS with 0.5% Tween 20

2.2.3.5. Coomassie-blue staining of SDS-PAGE gels

Coomassie-blue (Coomassie Brilliant blue R 250) is an irreversible, unspecific protein stain, suitable for staining of SDS-PAGE gels for the detection of differences between a particular protein sample and a control sample, when relatively high amounts of protein are present (microgram). Gels were incubated with the following staining solution for 15 min, destained with 10% acetic acid for 2 h and washed with H₂O.

Staining solution:

25% isopropanol

10% acetic acid

0.05% Coomassie-blue R-250.

2.2.3.6. Silver staining of polyacrylamide gels

The silver staining of the gels was made according to the producer's protocol. Silver stain Plus, Bio-Rad.

2.2.4. Cell culture

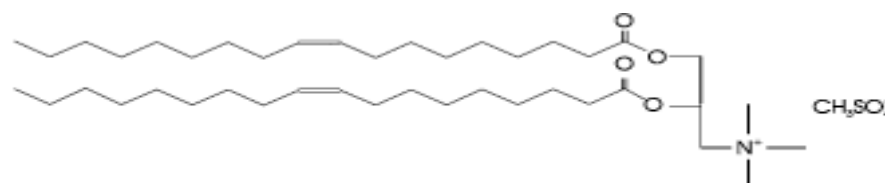
All experiments and treatments of cells were performed under sterile conditions using the sterile hood. Solutions and media were sterilized either by autoclaving or filtering, and, were pre-warmed before use (in a 37°C water bath). All glassware items, such as pipettes and bottles were autoclaved before use, and rinsed with 70% ethanol before taken under the sterile hood.

2.2.4.1. Basic handling of the cells, cell passage

Cells were trypsinized and resuspended in complete DMEM medium, and, split one to eight just before confluence was reached. Normal culture medium was 10% FCS/ 2 mM glutamine/20U Pen/20 µg Strep (penicillin and streptomycin) from *Bio*Whittaker* in 500 ml

For stocks: Cells were washed with PBS; treated with trypsin (500 μ l pro culture dish); scraped; centrifuged at 700 rpm for 5 minutes, and then the supernatant was removed. The pellet was resuspended in 80% DMEM /10% DMSO /20% FCS and the cells were transferred immediately on ice in cryotubes, and placed into the liquid nitrogen tank in which they can be kept for years.

HEK293 cells were transfected using the DOTAP method. DOTAP is a liposome formulation of a monocationic lipid (N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methylsulfate). Mixing DOTAP Liposomal Transfection Reagent with DNA results in spontaneously formed stable complexes. These complexes were added to the tissue culture medium (with or without serum) and the resulting mixture was added to the cells. These complexes adhere to the cell surface, fuse with the cell membrane, and then release the DNA into the cytoplasm. This method of DNA transfer is very gentle, without cytotoxic effects, so that the cells could be transfected with high efficiency.



One hour before transfection, the cells were transferred to 2% FCS DMEM. 5 µg of plasmid DNA were used for each 9 cm diameter culture dish. The DNA was diluted in DMEM. At the same time, 32 µl DOTAP were diluted in DMEM to a final volume of 100 µl, and both solutions were mixed without vortexing. After 10-15 minutes of incubation at room temperature, the DNA/DOTAP mixture was added to the cells (1x10⁶ cells/9 cm plate) in serum-free medium. 6 to 8 hours later, the medium was replaced with 10% FCS-containing medium. After 48 h the cells were collected and lysed.

2.2.4.3. Cell lysate preparation

For western blot and immunoprecipitation analysis of the expressed protein, cells were lysed and the soluble fraction was collected. Cells were harvested 48 h after transfection. Plates were washed twice with 2-3 ml of ice cold 1 x PBS buffer. 1 ml of lysis buffer was then applied to each 9 cm plate. Cells were scraped with rubber scraper, incubated for 10 minutes on ice and the cell suspension was transferred to Eppendorf tubes standing on ice. The suspension was centrifuged at 12000 g, at 4°C for 10 min and the supernatant was recovered and kept at -20°C.

Lysis buffer

50 mM Tris, pH 8.0

0.1% TritonX-100

80 mM NaCl

Protease inhibitor cocktail (Sigma).

2.2.4.4. Mouse brain membrane extract

Membrane protein extracts prepared from brains of mice were used for the co-immunoprecipitation experiments. Mice were sacrificed by cervical dislocation and the brain was collected and stored frozen at -80°C or directly homogenized in 10 ml of homogenization buffer plus fresh protease inhibitors (see below) with a sonicator by 4 times 20 s pulses. Cellular debris was removed by ultracentrifugation at 150000 g at 4°C for 60 min. The pellet, which contained membranes, was resuspended in 10 volumes extraction buffer (see below) plus protease inhibitors with a sonicator 4 times 20 s pulses. The extract was centrifuged at 150000g, at 4°C for 60 min. The supernatant, the final mouse brain membrane extract, was saved, aliquoted and stored at -80°C. Protein content (Bradford) and cholinesterase activity (Ellman) was determined in different fractions. The buffer was then changed by dialysis with PBS-T, to minimize the high salt content interference with the binding studies.

Homogenization buffer

10 mM Tris-HCl, pH 7.4

1 mM EDTA

0.02% NaN₃

Protease inhibitors (Sigma)

Extraction buffer

20 mM Sodium-phosphate, pH 7.0

1 mM EDTA
1 M NaCl
0.5% Triton X-100
0.02% NaN₃
Protease inhibitors.

2.2.4.5. Co-immunoprecipitation

Co-immunoprecipitation is a biochemical technique commonly used to demonstrate *in vivo* protein-protein interactions. If two proteins are interacting *in vivo*, then the direct immunoprecipitation of one protein with an antibody specific to that protein should precipitate other associated proteins.

Transfected HEK293 cells were lysed in PBS with 0.1% Triton X-100 plus complete protease inhibitors. The cell lysate (1 ml) was then incubated with anti-AChE or anti-Laminin antibodies (5 µg) at 4°C o/n, followed by incubation with Protein A/Sepharose for 1 h at 4°C. Likewise, the brain membranes fraction (1 ml) was subjected to immunoprecipitation by anti-AChE or anti-Laminin antibodies. Beads were washed six times by PBS plus 0.1% Triton X-100, and the proteins were eluted by 1x SDS sample buffer (30 µl). The associated proteins could be visualized by Western blot analysis.

2.2.5. Stainings

2.2.5.1. Preparation of mouse brains and chicken retinas for cryostat sectioning

For morphological applications on mouse brains, the brain and retina were fixed and cryoprotected. Preparation methods varied, according to the age of the mouse and the desired fixation. Mice at age day 3 (P3) or older were anaesthetized with chloroform and perfused. Through perfusion, the blood of the animal was removed and the whole mouse body was fixed. PBS was used to remove the blood. For the majority of the applications, 4% paraformaldehyde in 0.1 M PBS was used for fixation. After perfusion, the brain was collected, kept in fixative at RT for 2 h and then in 25% sucrose at RT o/n. The sucrose incubation (cryoprotection) protects the brain from the low temperatures used in cryostat sectioning, by increasing its osmolality. When not used immediately after cryoprotection, the brains were kept in 25% sucrose at 4°C.

Chicken embryos at different embryonic stages were isolated from eggs, decapitated and the eyes were enucleated. Following 3 wash steps in PBS (5 min), the eyes were fixed

in 4% formaldehyde o/n at 4°C (E9-E19) or 1 h at RT (E4-E8). The fixative was removed and the eyes were transferred into 25% sucrose. The eyes were let in the sugar solution until they completely sunk.

2.2.5.2. Cryostat sectioning

Brains and eyes were covered with embedding Tissue Tek and frozen at -20°C - -30°C. After freezing, they were equilibrated at -22°C. Sectioning was carried out with chamber temperature -25°C and object temperature -22°C. Sagittal or coronal sections were cut. For most immunohistochemical applications, 20 µm sections were cut. Retinal sections were 10 µm thick. Sections were mounted directly on glass slides with 0.5% gelatin and assayed on them.

2.2.5.3. Immunohistochemistry

Immunohistochemical staining was carried out on mouse brain sections in order to co-localize AChE with its binding partners. The general protocol is given below. However, this protocol was quite often modified in one or more of its parameters, or slightly different protocols were applied in parallel, in an attempt to optimize staining.

General control methods

All antibodies and antisera were characterized by the companies or by the groups from which they were obtained. The specificity of immunolabelling was further verified by the omission of the primary antibody from the protocol. This resulted in a lack of immunocytochemical fluorescent signal upon microscopic examination. Varying dilutions of secondary antibody enabled the optimal concentration of fluorescence to be established in order to avoid background labeling.

General protocol

The sections were dried on a heating block and incubated in 0.5% Triton X-100 in PBS at RT for 15 min, shortly washed with PBS and blocked with 3%BSA in PBS at RT for 1 h. The primary antibody was diluted in 0.1% Triton X-100 PBS, and incubated at 4°C o/n (see results section for concentrations used). After 3 washing steps with PBS-T the sections were incubated with the secondary antibody 1:100, in PBS-T at RT for 30 min. The excess of unbound second antibody was washed 3 times with PBS-T and the sections were incubated for 1 min with DAPI (stains the nucleus). After 2 washing steps with PBS, the sections were dried on a heat block, and mounted in glycerine.

2.2.5.4. Karnovsky and Roots cholinesterase activity staining (1964)

The enzymatic activity is assayed by the formation of an insoluble brown precipitate (Hatchett's brown) that is visible on a light microscope. The retinal or brain cryostat sections were dried on a heat block at 37°C and incubated 2 times for 15 min in a 0.1 M Tris-Maleate buffer. After this step of equilibration, the sections were incubated for up to 4 hours in dark at 37°C in the following solutions mix:

0.1% ATC (substrate for AChE)

0.065 M Tris-Maleate buffer, pH6

0.1 M $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot \text{X}2\text{H}_2\text{O}$

30 mM CuSO_4

iso-OMPA 10^{-3} M

5 mM $\text{K}_3\text{Fe}(\text{CN})_6$

As control for the staining the above solution was used, but without ATC.

After the incubation time, the sections were washed 2 times 10 min in distilled water and dried on a heat block.

Buffers:

0.1 M Tris-Maleate Buffer pH 6

12.2 g Tris

11.6 g Maleic acid.

2.2.5.5. *In situ* hybridisation on cryostat sections

Digoxigenin probe synthesis was made using the DIG RNA-labeling kit from Roche. The *in situ* hybridization procedure can be split in 5 main steps:

1. Hybridization

The sections were defrosted at room temperature (about 1 hour) and in this interval the riboprobes were diluted in hybridization buffer (0.1-1 µg/ml) and denatured for 10 min at 70°C. 150 µl probe were added to each section and the slides were covered with a 22 x 50 mm sterile coverslip. The probes were hybridized o/n at 65°C in a sealed box with 2XSSC and 50% formamide.

Hybridization buffer: 1 ml 10X Salts, 5 ml formamide, 2 ml 50% dextrane-sulfate, 1 ml yeast-RNA, 100 µl 50X Denhardts, 900 µl H_2O .

50% Denhardts: 1% BSA, 1% Ficoll, 1% polyvinylpyrrolidone.

2. Posthybridization

The slides were transferred to a slide rack and incubated at 65°C in washing solution (see below) for 10 minutes. The coverslips should normally fall off the slides at this step. After the first wash interval, two more wash steps followed, both of them for 30 minutes in washing solution at 65°C. The sections were then equilibrated in another buffer, MABT, 2 times for 30 min at RT.

Washing Solution: 1x SSC, 50% formamide, 0.1% Tween-20, pre-warmed to 65°C.

MABT: 100 mM Maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween-20.

3. Blocking and antibody staining

The solution on the slide was removed and the sections were blocked in 20% heat inactivated goat serum in MABT for at least one hour at RT, without a coverslip. After the blocking, the sections were stained with 100µl anti-DIG alkaline phosphatase 1/2000 to 1/5000 diluted in MABT + 20% heat-inactivated goat serum, for o/n at RT in a humid chamber.

4. Post antibody washes

The sections were washed at RT in MABT for 1 hour each 2-3 times and after with the AP-buffer for 2 times 10 minutes at RT. After washing the unbound antibody, the alkaline phosphatase substrate was given (100µl of 1% NBT/BCIP in AP-Buffer) and incubated for a few hours or o/n in dark at RT.

AP-Buffer: 0.1M Tris, pH 9.5, 0.05M MgCl₂, 0.1M NaCl, 0.1% Tween-20.

5. Staining reaction

The staining reaction was stopped by washing with PBS 2 times 30 minutes at RT. The sections were embedded in Kaisers Glycerin-gelatine (60 µl) and coverslips were added on the slides.

2.2.5.6. Microscopy

The staining was viewed and documented on an Axiophot (Zeiss) microscope, equipped with fluorescence and a camera. The pictures were taken using the Kamcon software and processed using the Adobe Photoshop 7.0 software.

2.2.6. Surface plasmon resonance

This method was used primarily to identify and characterize non-covalent interaction between surface-immobilized AChE and a brain membrane extract. The BIAcore 3000 instrument was used. This instrument uses an optical method to measure the refractive index near (within ~ 300 nm) a sensor surface (see Figure 5).

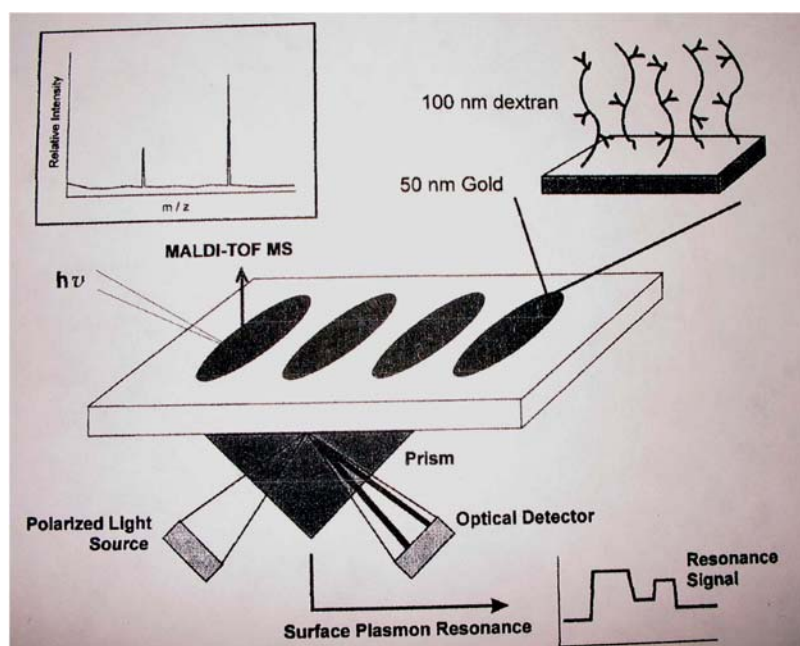


Figure 5. Illustration of the combined surface plasmon resonance – mass spectrometry approach. First, a derivatized biosensor chip, having four flow cells, was used in the analysis of interaction between surface-bound AChE and solution-ligand(s). The chips was removed from the BIAcore 3000 and inserted into the mass spectrometer.

In the BIAcore instrument the sensor surface was forming the floor of a small flow cell, 20-70 nl in volume, through which an aqueous solution passes under continuous flow (with a speed of 1-100 $\mu\text{l}/\text{min}$). In order to detect an interaction AChE was immobilized onto the sensor surface. The process of immobilization of AChE to the chip surface is called derivatization. Its binding partner(s) (unknown, possibly present in a brain extract) was injected in aqueous solution through the flow cell, under continuous flow. As the proteins bound to AChE, the accumulation of protein on the surface resulted in an increase in the refractive index. This change in refractive index is measured in real time, and the result plotted as response or resonance units (RU) versus time (sensogram). A response is also generated as a difference in the refractive indices of the running and sample buffer. This background response was subtracted from the sensogram. One response unit (RU) represents the binding of approximately 1pg protein/ mm^2 .

A CM5 (carboxylated dextran) chip was used. All four flow cells of the sensor chip were activated through exposure to N-hydroxysuccinimide [0.1 M prepared in 20 mM HEPES, 0.005% surfactant P20, 150 mM NaCl, 3 mM EDTA, pH 7.4, containing 0.1 M N'-ethyl-N'-(dimethylaminopropyl)carbodiimide], derivatized with AChE or neuroligin-1. The remained free carboxyl groups on the chip after derivatization were blocked by exposure to 1 M ethanolamine hydrochloride.

3. Results

PART I: Is neurexin-1 β a possible AChE binding partner?

Co-localization and binding studies.

Neurexins are relatively “new” molecules (Südhof *et al.*, 1995). At the beginning of this work in 2001 not too many histological localization studies about neurexins were published. Therefore, before starting the binding experiments, the retinal expression pattern of neurexin-1 β was analyzed by *in situ* hybridization and compared with the AChE expression pattern. The *in situ* hybridization method was not established in the lab until that time point. Therefore, the distribution of a series of other markers, e.g. synaptotagmin, neurofilament M, and tyrosine hydroxylase, was studied. These markers were used as controls to establish the accuracy and reliability of the method.

3.1.1. AChE and neurexin-1 β localization in the developing retina

The first step on the way to show the direct connection between AChE and neurexin-1 β was to show that they co-localize in the same CNS regions. Due to its highly organized layered structure of alternating neuronal cell bodies and neurites, the retina presents some advantages over other parts of the CNS for the study of developmental processes, especially synaptogenesis.

The expression pattern of neurexin-1 β mRNA was analyzed by *in situ* hybridization. A 256 bp long neurexin-1 chicken riboprobe was used (Patzke and Ernsberger, 2000). Embryonic chick retina sections, 10-15 μ m thick, corresponding to the E4 (E-embryonic day) up to E19 were hybridized with the above mentioned fragment. As control for the specificity of binding, a fragment synthesized in the ‘sense’-orientation was used. No signal was present on the sections incubated with this fragment. Figure 7 shows retinas at different developmental stages hybridized with the neurexin probe. The neurexin-1 β staining revealed an early appearance of this transcript in the retina. The first signal for neurexin was detected at E6 (Figure 7E) in the ganglion cell layer. At E9, the neurexin-1 β transcripts appeared not only in the GCL, but also in the inner half of the INL (Figure 7H). The staining remained localized on the GCL and the inner half of INL (where most of the amacrine cells are localized) until the E19 stage (Figure 7K). E19 displayed the same expression pattern, except that here some cells situated at the border to the OPL (possibly horizontal cells)

were also labeled (Figure 7H). The absence of a commercially available antibody against chicken neurexin limited the expression studies to the mRNA level.

In parallel, retinas from E4 to E19 were stained with an anti-AChE antibody (clone 3D10, Tsim *et al.*, 1988). The omission of the primary antibody from incubations resulted in the absence of immunolabeling within the retina. Figure 7A, C, F, I, and L, shows 3D10 labeled neurons which are starting to differentiate and later perform synaptogenesis. The first cells to be stained are ganglion cells, followed by amacrine cells situated in the INL. The strong AChE labeling in the IPL is associated with the formation of synaptic connections. Karnovsky-Roots activity staining was performed on the same set of retinal developmental stages. The staining was not only consistent with histochemical protocols for AChE, but also with the previously described immunostaining, being found in ganglion, amacrine and horizontal cells and in 2 bands in the IPL (Millar *et al.*, 1987). The Karnovsky-Roots technique is a procedure based on the AChE activity. This assay stains just the active protein in a tissue and thus provides more information related to the presence of an active or inactive enzyme. The same cell populations labeled with 3D10 were also histochemically stained with the Karnovsky-Roots method, showing that during retinal development AChE is expressed in an enzymatically active form.

By comparing the neurexin-1 β expression pattern with AChE, it could be concluded that the neurexin-1 β labeled cells are also AChE labeled, and temporally neurexin-1 β follows with a 2 days delay the AChE expression.

Standard abbreviations:

RPE- retinal pigment epithelium,

ONL- outer nuclear layer

OPL- outer plexiform layer

INL- inner nuclear layer

IPL- inner plexiform layer

GCL- ganglion cell layer

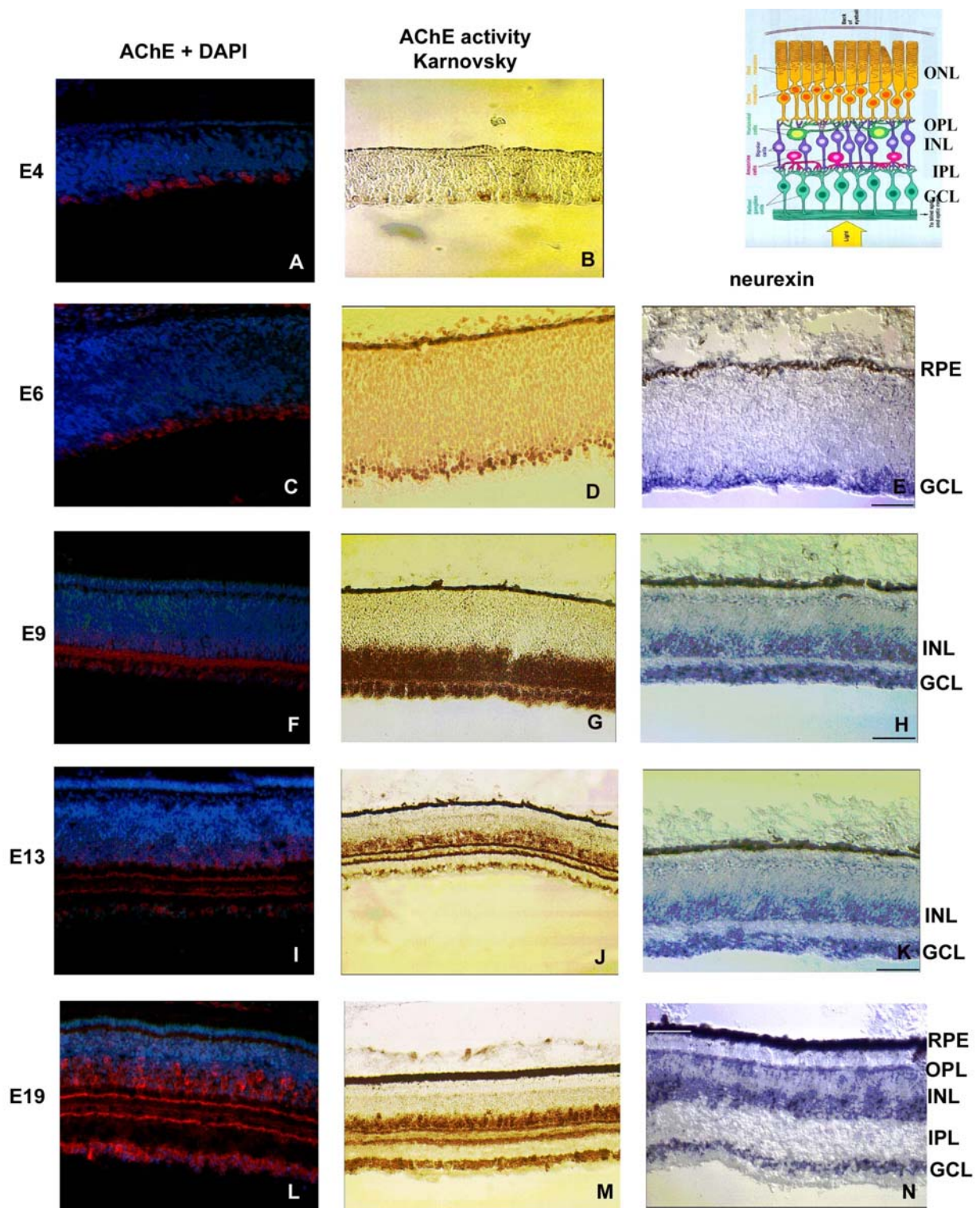


Figure 7. Localization of AChE and neurexin-1 β in the developing chicken retina. (A), (C), (F), (I), (L) are retinas stained with a mouse anti-AChE chicken antibody (red) and DAPI (blue). (B), (D), (G), (J), (M) show a Karnovsky-Rosenthal AChE activity staining at stages E4, E6, E9, E13, and E19, respectively. (E), (H), (K), (N) are retinas probed with a neurexin-1 β fragment by *in situ* hybridization. Scale bar 100 μ m.

3.1.2. Chicken retinas display synaptotagmin I distribution similar to neurexin-1 β

To have a further control of the neurexin-1 β expression pattern, the distribution of synaptotagmin I in the retina was investigated by *in situ* hybridization. Synaptotagmin I is a synaptic vesicle protein that binds Ca^{++} ions, and forms complexes with the cytoplasmic domains of neurexins (Petrenko *et al.*, 1991). Therefore, the neurexin expression pattern should overlap synaptotagmin's expression pattern and relate to the same cell populations. Figure 8 shows an assembly of different retinal developmental stages used to hybridize with a DIG-labeled synaptotagmin RNA probe (Campagna *et al.*, 1997). By comparing Figure 8A with Figure 7E it can be seen that starting with the same embryonic day, synaptotagmin I is indeed expressed in the same cells as neurexin-1 β . The synaptotagmin transcript "follows" the neurexin-1 β transcript throughout retinal development (see Figure 7H, K, N, and Figure 8C, E, G, respectively). The first signal appeared at E6 and was localized in the ganglion cell layer (see Figure 8A). At E11, when the IPL is already formed, the synaptotagmin positive cells split into 2 populations: the ganglion cells and cells situated in the inner half of the INL. These 2 populations continued to be labeled until E19. Figure 8E and 8G show that besides the above described cell populations there is also a synaptotagmin I signal in the outer segments of photoreceptors. This was surprising, yet cannot be explained as a staining artifact, since hybridizing with a 'sense' probe would give no signal.

The results showed that the synaptotagmin I expression pattern overlaps with neurexin-1 β expression in the chicken developing retina.

3.1.3. Neurofilament M – a possible ganglion cell marker in the chick retina?

The differentiation of a retinal progenitor into a mature cell requires the expression of a large number of molecules. These molecules can serve as markers for the study of developmental steps. Therefore, expression studies for neurofilament M were performed in the chick retina. Sections of different retinal stages were probed with a 553bp chicken neurofilament probe (Stanke *et al.*, 1999). Figure 9 shows that starting with E6 up to E19 the neurofilament M gene expression could be found exclusively in the ganglion cell layer. This localization, distributed in a constant manner in the developing retinas suggested that neurofilament M should be considered a marker for the ganglion cells. Nevertheless, immunostainings with an antibody against chicken neurofilament M should also be done, in order to localize the protein in the retina.

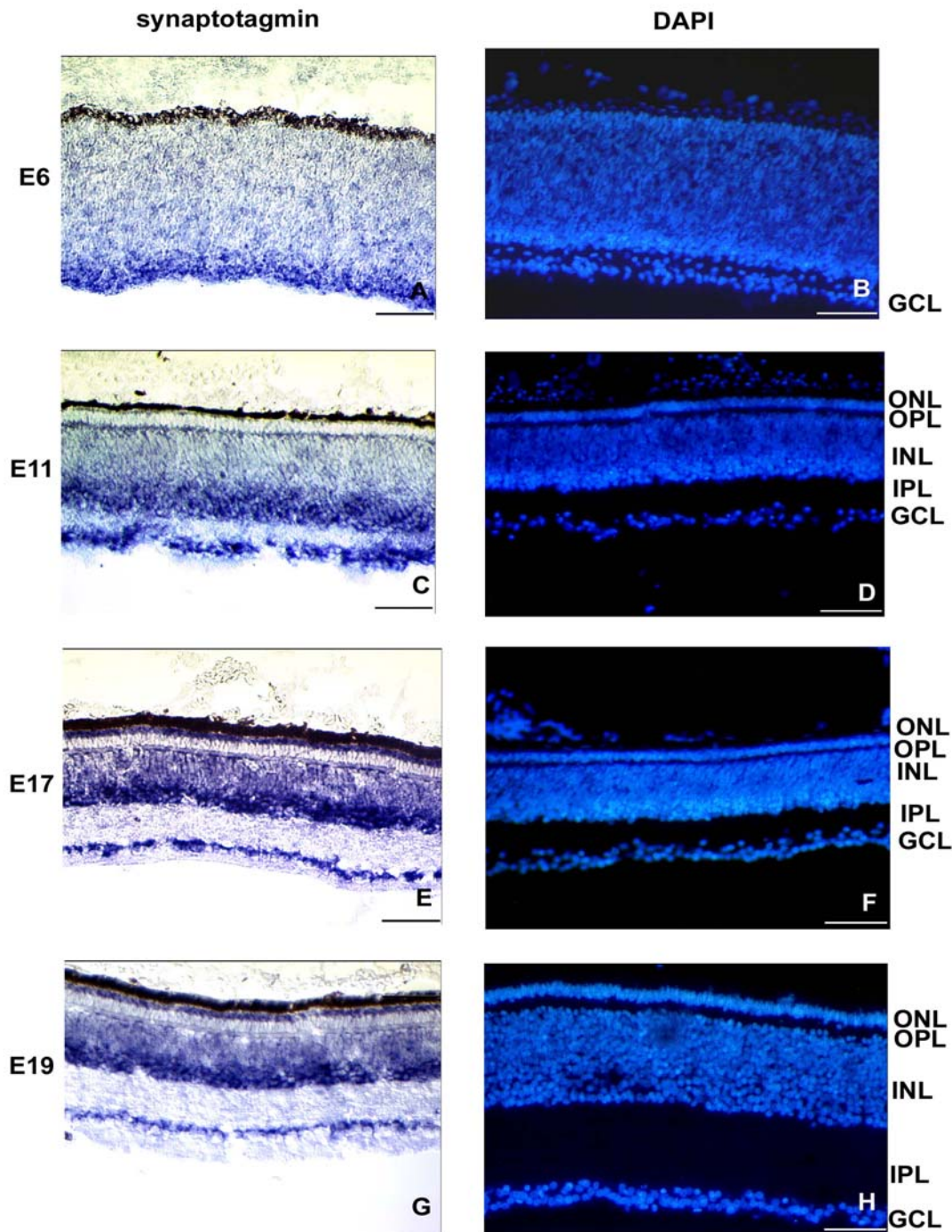


Figure 8. *In situ* hybridization with a chick synaptotagmin I probe. (A), (C), (E), (G) retinas corresponding to E6, E11, E17, and E19, respectively, stained for synaptotagmin (blue). E- Embryonic day. (B), (D), (F), (H) represent DAPI staining of the same developmental stages. ONL- outer nuclear layer, OPL-outer plexiform layer, INL-inner nuclear layer, IPL-inner plexiform layer, GCL-ganglion cell layer. Scale bar 100 μ m.

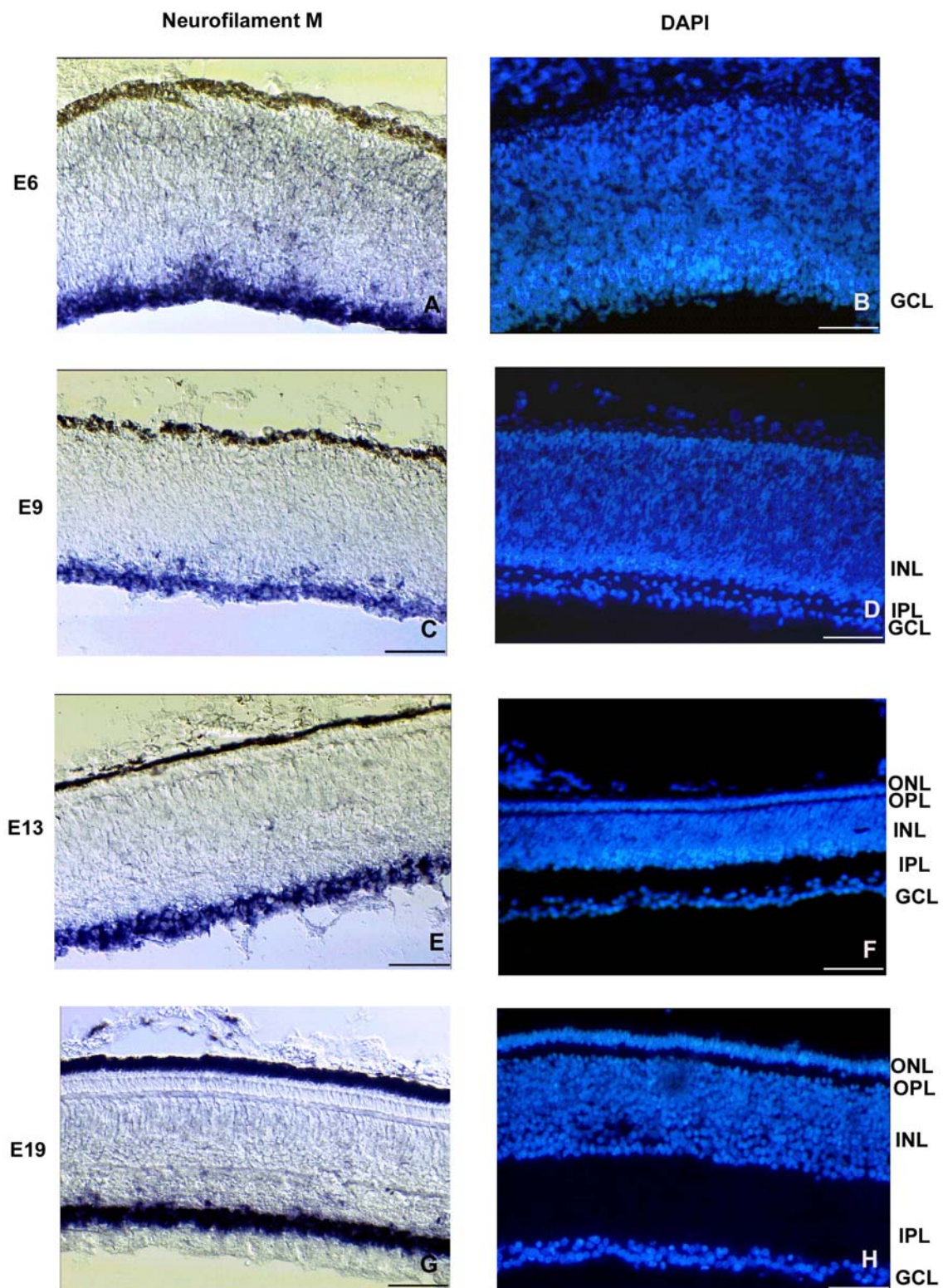


Figure 9. *In situ* hybridization with a neurofilament M probe. (A), (C), (E) and (G) show retinas hybridized with a neurofilament M riboprobe. The labeled cells are blue. (B), (D), (F) and (H) represent retinas stained with DAPI. Cell nuclei are blue. Scale bar 100 μ m.

3.1.4. Tyrosine hydroxylase (TH)

When the levels of ChAT (choline acetyltransferase) and ACh were compared with the activity of AChE, the AChE activity was much higher. It is known that there are potentially other sources of AChE except the cholinergic neurons, including GABAergic terminals, or even GABAergic neurons (Henderson and Greenfield, 1984). Tyrosine hydroxylase is a marker for both noradrenergic and dopaminergic neurons, since it is the key enzyme in the production of these two neurotransmitters. *In situ* hybridizations with a chicken TH riboprobe were performed mainly as a positive control for the *in situ* hybridization procedure. Chick TH cDNA was a kind gift of Dr. Uwe Ernsberger (Ernsberger *et al.*, 1995). It is already documented in mice (Haverkamp and Wässle, 2001) that TH is expressed in a specific population of amacrine cells in the retina, situated at the INL border with the IPL. In the chicken retina, the same distribution as the one described in the literature was observed. Just about 7 - 10 cells/retina were TH positive. Figure 10 illustrates these cells and their morphology and position.

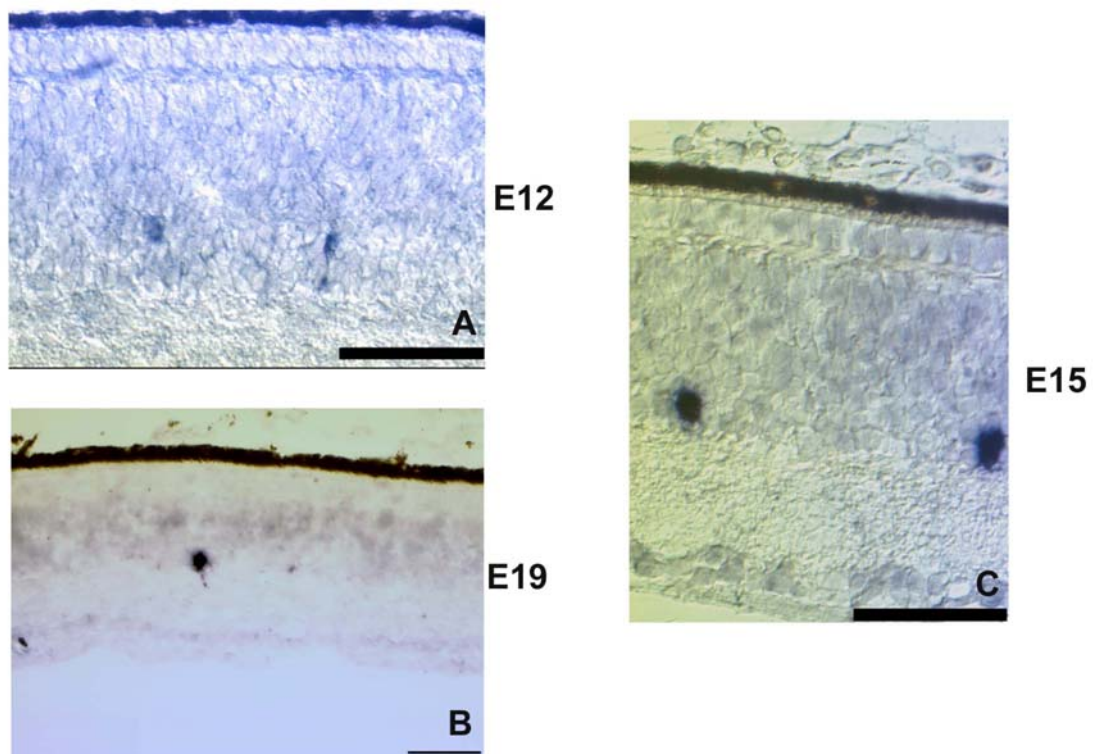


Figure 10. TH-positive neurons in the chicken retina. The labeled cells are blue. Scale bar 100 μ m.

3.1.5. On the way for finding an AChE interaction partner – surface plasmon resonance experiments

The co-localization of AChE and neurexin-1 β in the retina are not sufficient evidence that the two proteins indeed do interact. Therefore, interaction studies were to be done. These studies were stopped because Prof. Taylor (UCSD) shared unpublished information and later published experiments that showed that AChE does not bind to neurexin-1 β in the experimental system used (Comoletti *et al.*, 2003). The system that they used to study the binding was based on the surface plasmon resonance, an *in vitro* method that allows detection of binding between pg amounts of protein. However, this does not exclude the possibility that AChE binds to neurexin-1 β *in vivo*. Therefore, the use of other experimental approaches, e.g. co-immunoprecipitation, can bring new insights into the AChE-neurexin relation. As cell adhesion molecules, neurexins are known to be expressed in hundreds of isoforms. This leaves open the possibility that one neurexin isoform might interact with AChE during development. Nevertheless, the study of these possible interactions represents a complex project with an outcome much too unsure for a PhD work.

The aim of the project became then the search for an unknown binding partner for AChE that should have a synaptic localization. A first attempt was made using **biomolecular interaction analysis mass spectrometry** (BIA/MS). This is a two-dimensional analysis technique. In the first dimension the surface plasmon resonance (SPR) is used to detect the binding molecules and in the second dimension these molecules can be analyzed by mass spectrometry.

3.1.6. Establishing a protocol for sample recovery from a BiAcCore chip for mass spectrometry

In order to identify binding partners for AChE, the first step was to immobilize AChE on a Biacore CM5 chip and to flow a brain extract over this chip. The proteins bound to the chip (and so to AChE), were recovered in a buffer and further analyzed. The Biacore 3000 Software includes a microrecovery function designed for recovering material from the sensor surface in a very small volume. A protocol had to be established. For this procedure neurexin-1 β and neuroligin-1 were used. A direct binding of these two proteins was already documented (Ichtchenko *et al.*, 1995, Ichtchenko *et al.*, 1996, Comoletti *et al.*, 2003). Neuroligin was covalently immobilized on a CM5 chip (carboxymethyl dextran surface) through its amino groups, following the standard coupling protocol with EDC/NHS - N-ethyl-N'-(dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (Johnsson *et al.*, 1991). With

this procedure the carboxylated dextran is activated, the protein is covalently bound to the chip through amine/NHS displacement; the remaining NHS in solution is later blocked by exposure to ethanolamine. Figure 11 shows that there was a response of 8500 resonance units (Δ RU - response units) which amounts to the immobilization of 86 pmol neuroigin (1000 RU represents ~ 1 ng protein). This amount of neuroigin-1 bound to the chip is considered to be a high-density ligand surface. Neurexin-1 β was diluted in 0.01M HEPES, 0.15M NaCl, 3 mM EDTA, 0.005% P20 surfactant (HBS-EP) and injected over the neuroigin-1 flow cell with a constant flow rate of 1 μ l/min.

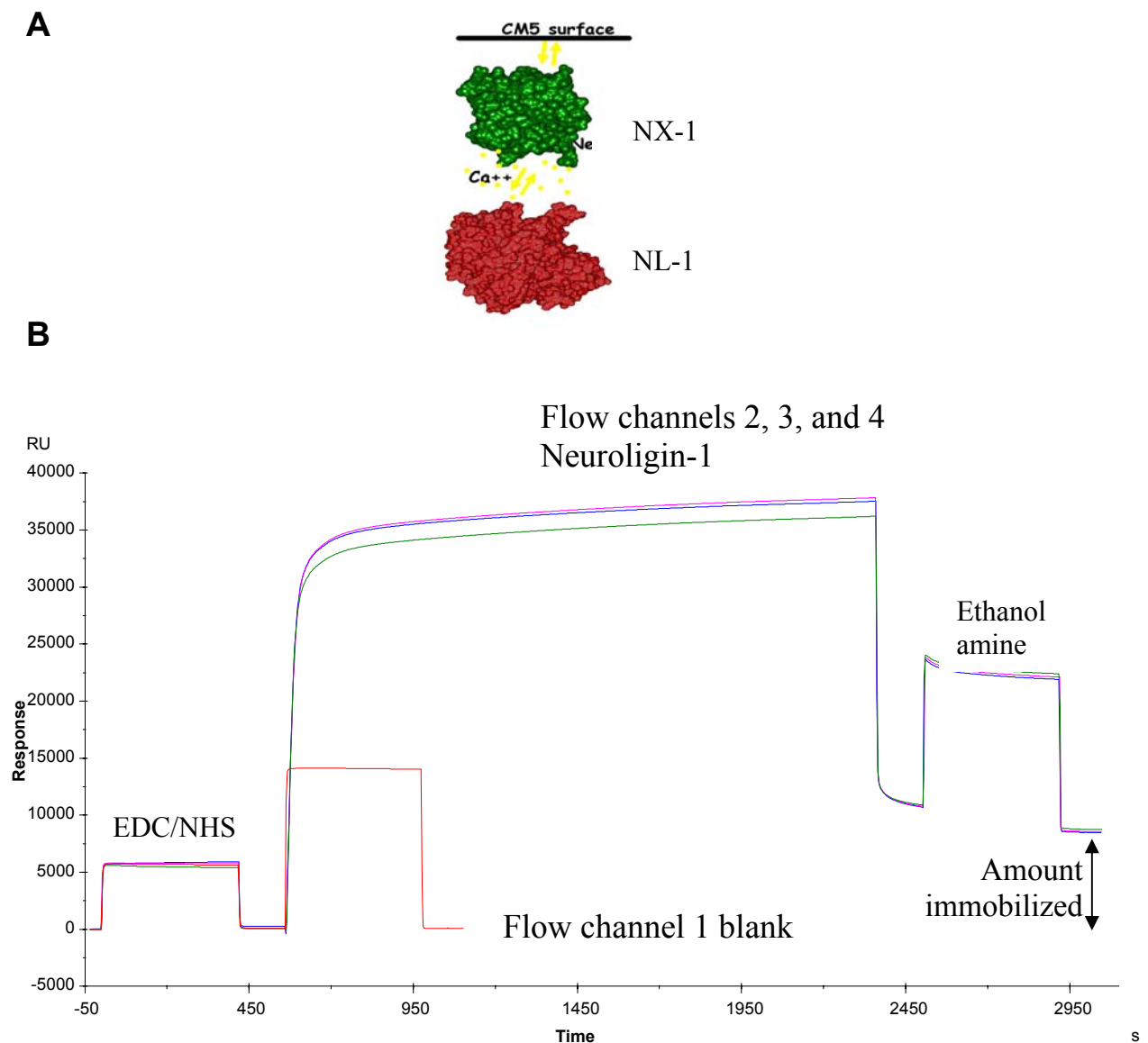


Figure 11. Neuroigin-1 immobilization on a CM5 chip. (A) Schematic representation of the experimental design. **(B)** After the activation of the carboxymethyl matrix with a 35 μ l injection of EDC/NHS, a 200 μ l aliquot of neuroigin-1 was injected over the second, third and fourth flow cell (green, blue and purple line), followed by 35 μ l ethanolamine injection. The amount of immobilized neuroigin-1 corresponds to a Δ RU=8500. RU - resonance units.

Figure 12 shows the binding of neurexin-1 β to neuroligin-1 and also the amount of neurexin recovered. Using the program illustrated in Figure 13 it was possible to recover the bound neurexin-1 β in a total volume of 7 μ l buffer (HEPES). This volume was re-injected on the CM5 neuroligin channel. This re-injection had two purposes:

- to determine if there was any neurexin-1 β recovered (this showing a binding to neuroligin-1);
- to verify how much from the injected neurexin-1 β was recovered.

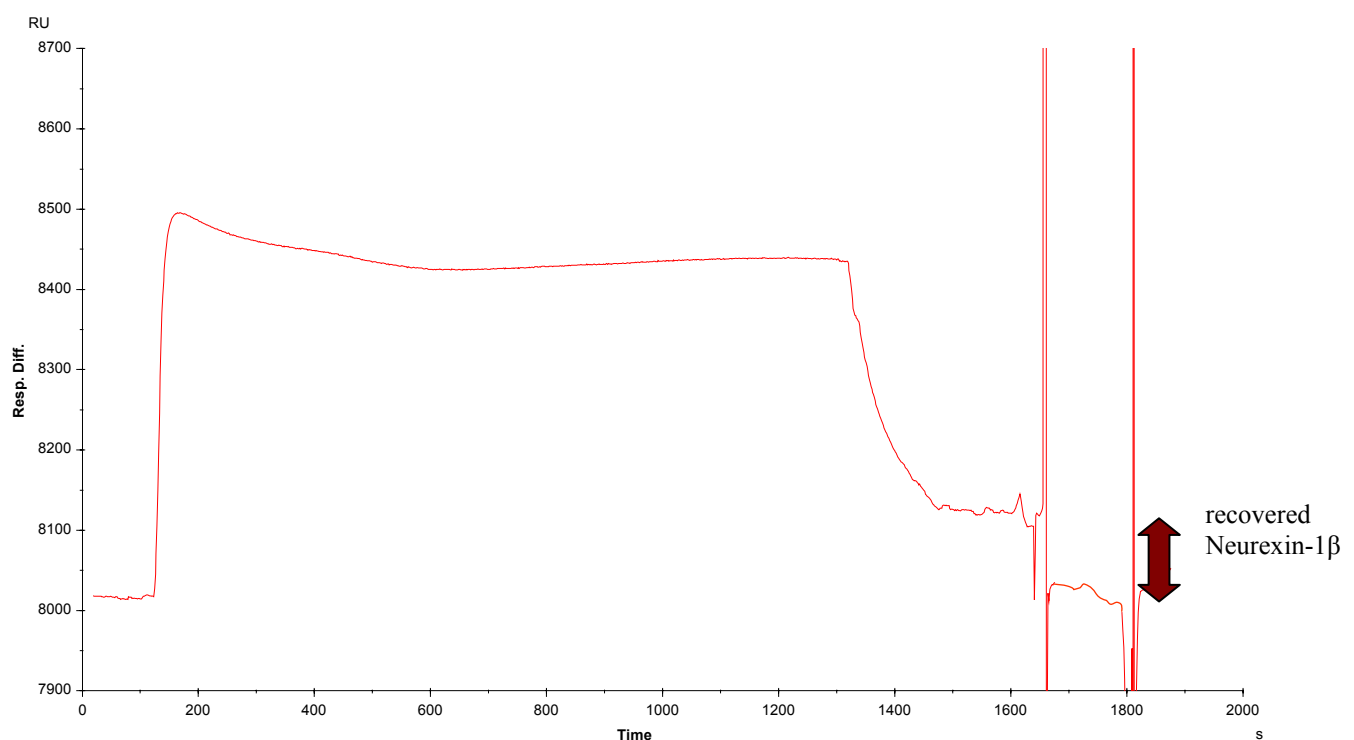


Figure 12. Sensogram resulting from the injection of 20 μ l 1 μ M neurexin-1 β over the surface of a neuroligin-1-derivatized flow cell. Δ RU=140 indicated the retention of 140 pg neurexin-1 β . The bound material was recovered by injecting 7 μ l HEPES using the program illustrated in Figure 13.

Microrecovery method

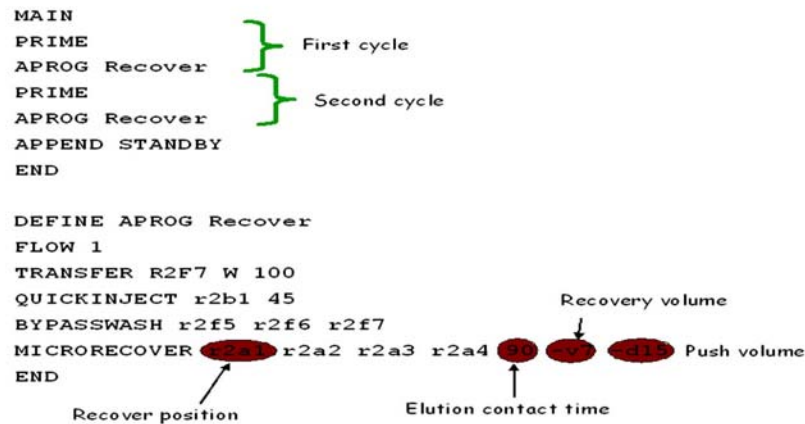


Figure 13. Program developed to recover bound material from a BIAcore AChE derivatized chip.

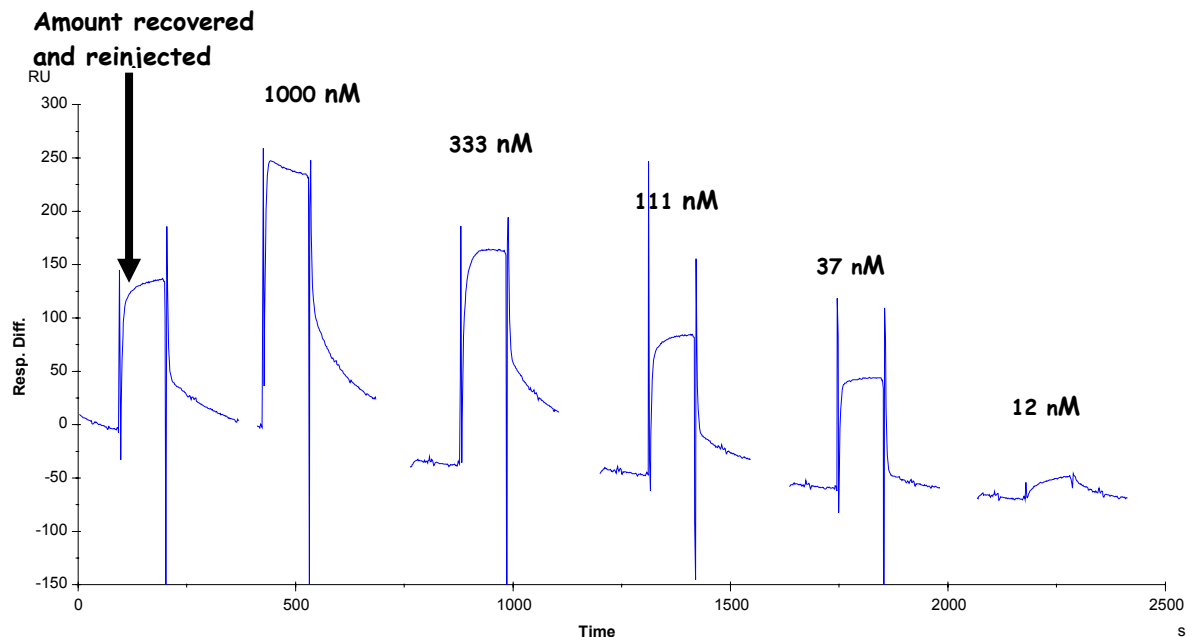


Figure 14. Determination of the amount of neurexin-1 β recovered through comparison with a series of standard neurexin concentrations.

The 140 pg material obtained by injecting neurexin-1 β over a neuroligin surface (see Figure 12) were recovered and reinjected over the same surface. The same Δ RU=140 was obtained (see Figure 14). Through comparison with a series of standard neurexin

concentrations, the amount of neurexin-1 β recovered was estimated to 150 nM (see Figure 14). The recovery procedure was established with a minimal loss of the material bound and therefore further used for AChE ligand fishing.

3.1.7. Using the surface plasmon resonance to find AChE binding partners

Using the protocol described in the 3.1.6., a sufficient quantity of mouse AChE (100 fM) was immobilized to a CM5 sensor surface (sensogram not shown). A 50 μ l brain extract aliquot was injected with a very low speed 1 μ l/min over the 3 flow channels derivatized with AChE. Figure 15 shows the sensogram resulted from this injection.

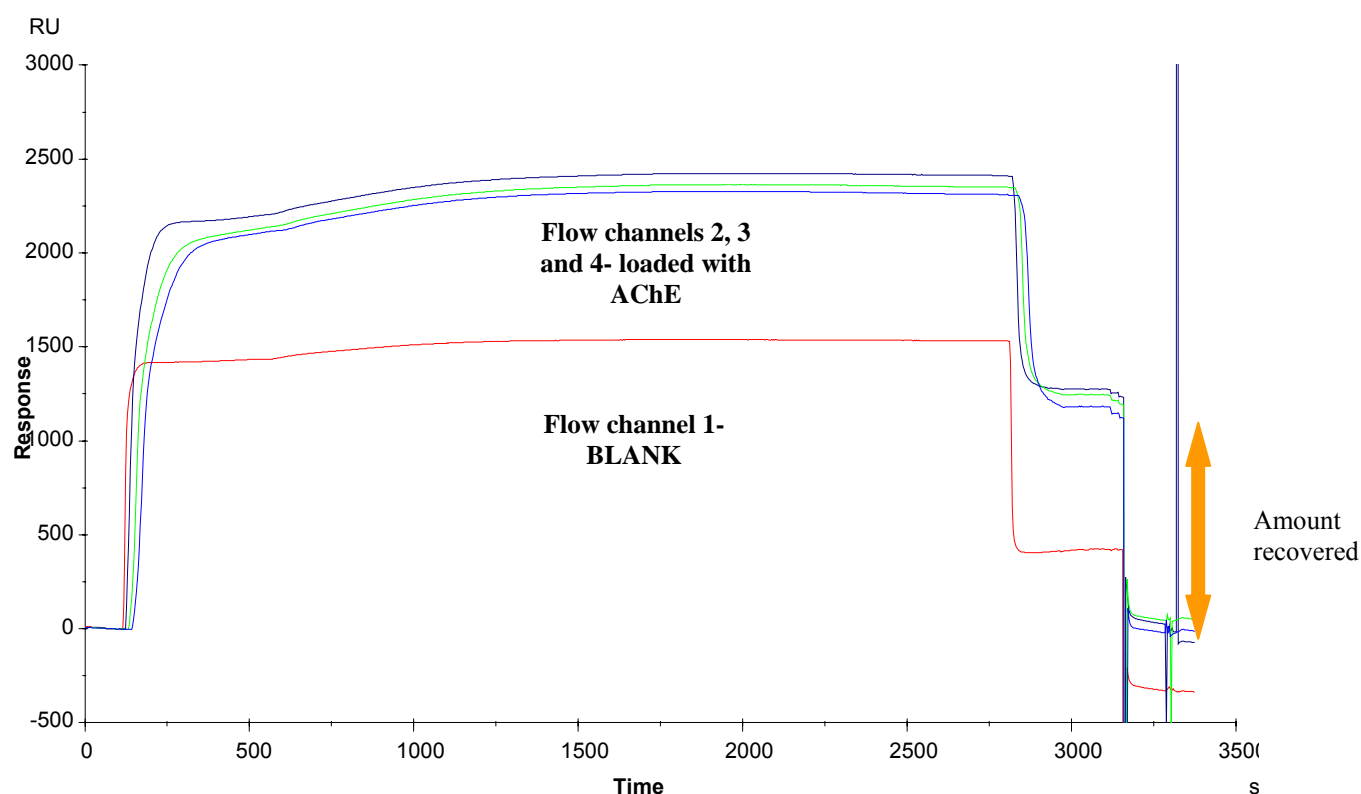


Figure 15. Sensogram resulting from the injection of a 50 μ l brain extract over the surface of an AChE derivatized flow cells.

A specific binding of 1350 response units was observed which corresponds to 1.35 ng protein. These results showed that there were proteins that specifically or nonspecifically bind to AChE. To gain some information about the nature of the fished AChE ligand(s), a recovered aliquot was analyzed by SDS-PAGE and silver staining. Figure 16 shows the silver staining of the proteins bound to an AChE derivatized chip and recovered using the established protocol (3.1.6.), separated on a SDS-gradient gel (4%-20%).

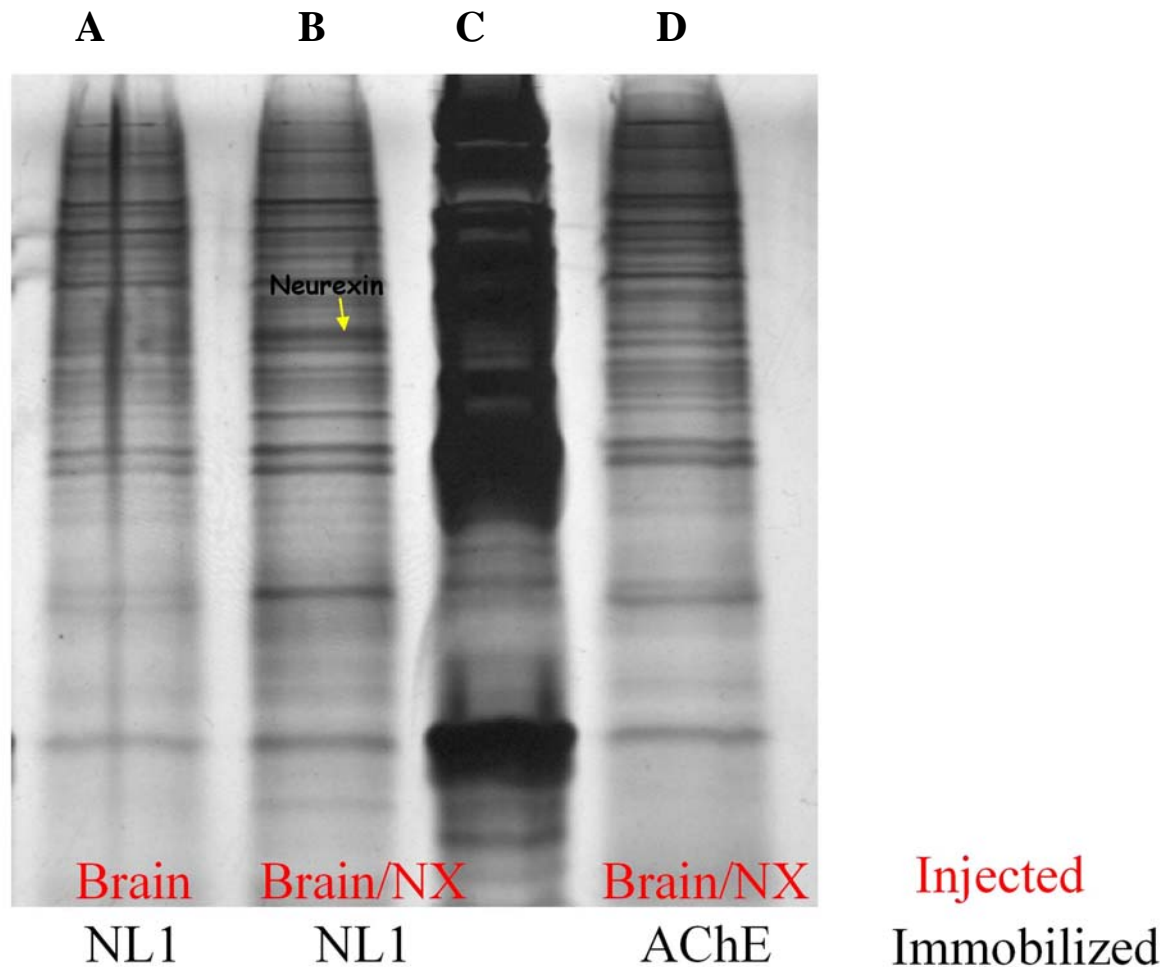


Figure 16. Silver staining of the material recovered from BIAcore chips. (A) Material recovered after the injection of a brain extract over a neuroligin-1 derivatized surface, subjected to SDS-PAGE followed by silver staining. **(B)** Material recovered after the injection of a brain extract including 1 ng purified neurexin-1 β over a neuroligin-1 derivatized surface. **(C)** Molecular weight marker. **(D)** Material recovered after the injection of a brain extract including 1ng neurexin-1 β over an AChE derivatized surface.

Figure 16, lane A, shows that a brain extract was used for establishing if neuroligin-1 detects its ligand, neurexin-1 β , in a complex biological mixture and at very low concentrations. In another test, to the brain extract was added purified recombinant neurexin-1 β (Figure 16, lane B). This would serve as control for the procedure, in view of the fact that neuroligin-1 should be able to recognize its ligand from a complex biological mixture. Figure 16, lane C illustrate the proteins that were bound to an AChE-derivatized surface. As the results show, there was a lot of background binding to the flow channels, independent of the protein derivatized on the chip. Very few significant differences were observed between the 2 flow channels (see Figure 16A, B and C).

In conclusion, a pilot investigation was carried out using BIAcore analysis to establish if AChE interacts with proteins from a mouse brain extract. AChE was loaded onto a CM5 chip. A brain extract was passed across the flow-cells containing AChE. Significant association was observed. However, the analysis of the bound material revealed background binding.

PART II: Screening for AChE interaction partners with the yeast two-hybrid system

The combination of surface plasmon resonance (SPR) with MALDI-TOF mass spectrometry (matrix assisted laser desorption/ionization time-of-flight) proved to be a good alternative for searching for AChE binding partners. The method does not raise the problem of using high amounts of purified protein like other biochemical approaches. In addition, a pre-clearing of the brain lysate by an affinity chromatography step with AChE immobilized on a column reduced the background binding in the surface plasmon resonance assay (not shown). However, the interactions happen in an *in vitro* assay, in buffers selected by an experimentator. Therefore, a genetic approach, namely yeast two-hybrid, was chosen to look for AChE binding partners. Yeast systems are generally intensely optimized and are closer to an *in vivo* system.

3.2.1. AChE as bait for the yeast two-hybrid screen

3.2.1.1. Cloning of the AChE constructs for the yeast two-hybrid screen

To clone the bait into the pVJL11, a yeast vector that contains the LexA BD, a soluble, monomeric form of mouse acetylcholinesterase (mAChE), was used (Marchot *et al.*, 1996). This form, which was truncated at its carboxyl-terminal end, was generated from a cDNA encoding the AChE by insertion of an early stop codon at position 549. The cDNA contains the AChE exons 2, 3, 4, and 6, encoding the catalytic subunit of the mouse tetrameric and asymmetric form.

Due to the fact that some proteins can activate the reporter genes in the yeast two-hybrid system, several baits were constructed. Figure 17 shows the cloned AChE baits and the strategies used for cloning (PCR combined with restriction enzyme digestion and ligation). The approach was to create long AChE baits that cover extensive parts of the molecule, if not the entire AChE. It was shown that some cell adhesion molecules possess an 'esteratic domain', homologous to AChE. The homology to AChE was spread almost all over the molecule and not restricted to some short fragments (Cousin X. *et al.*, 1997). Therefore, a long AChE fragment including the catalytic (aa 234, 365, and 478) and the peripheral anionic site was chosen as bait and named pVJL11-N583A (N – N-terminus, aa 32-614, A-AChE), see Figure 17. Other shorter fragments (pVJL11-N472A, pVJL11-N263A, and pVJL11-C97A) were also cloned into the pVJL11 vector, with the intention to be used

later in order to identify the part of AChE involved in interaction. Before proceeding with actual screening, the chimeric cDNA was checked for mutations through sequencing. All the sequence data showed no mutation of the AChE fragments and therefore, they were used as bait to screen a mouse brain cDNA library.

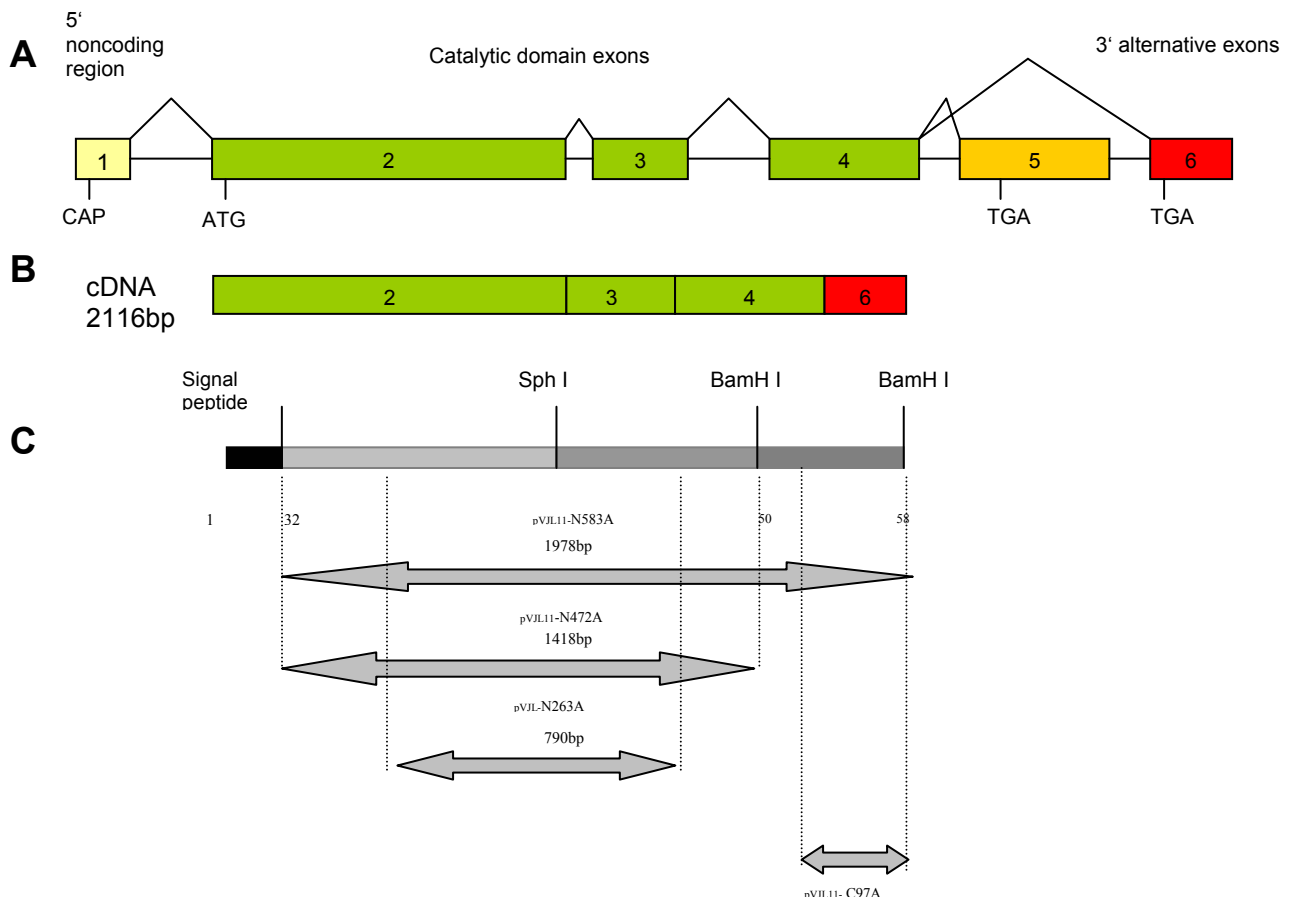


Figure 17. Cloning and sequence analysis of bait AChE. (A) The AChE comprises 6 exons: exon 1 is not translated, exon 2 has 1068 bp, exon 3 - 485 bp, exon 4 - 170 bp, exon 5 - 665 bp, and exon 6 - 328 bp. (B) The AChE cDNA used was 2116 bp length and the ORF was translated into a 581 aa long protein. (C) The bait constructed for the screening was the N-terminal 583 aa fragment of AChE (aa positions 32-614) derived from AChE cDNA and cloned in-frame with the LexA DNA binding domain (LexA BD) in the vector pVJL11 and named pVJL11-N583A (aa 32-614). Three plasmids encoding C- or N-terminally truncated baits were also constructed: pVJL11-N472A (aa 32-504), pVJL-N263A (aa 187-450), and pVJL11-C97A (derived from restriction and ligation of cDNAs or generated by polymerase chain reaction using a Pfu- proof reading DNA polymerase and primers that give the desired restriction sites). The sequences of all constructs were confirmed by sequencing.

3.2.1.2. Testing the Bait for auto-transactivation of the reporter genes (LacZ)

Since the yeast two-hybrid system is based on reconstitution of a functional transcription factor, testing the auto-activation capacity of the AChE is crucial for the overall feasibility. Approximately 5% of all proteins have some latent activating activity (Crickinge and Beyaert, 1999) and randomly generated fragments (like in libraries) even more so. Thus, the constructed BD-AChE fusion proteins were transformed into the L40 yeast strain and tested for the activation of the *HIS3* and LacZ reporter genes.

The activation of the *HIS3* was tested by growing the transformed yeast on DO/-Trp/-His plates. The lack of Trp in medium allowed just the growth of the yeast cells hosting the expression plasmid, and the lack of His allowed just the growth of the transformed yeast cells that were able to activate the reporter gene *HIS3*. A weak activation of *HIS3* was present on all the generated constructs in the form of a background growth. This growth was inhibited by adding 2.5 mM 3-AT (3-amino-triazole), a competitive inhibitor of the *HIS3* gene product. The amount of 3-AT necessary to inhibit the background growth was optimized by testing 3-AT concentrations between 1mM up to 15mM in medium. The lowest concentration that allowed after one week only small colonies to grow was 2.5 mM 3-AT, and therefore was further used for the screening. One has to consider that the use of too high concentrations could inhibit also the growth of the weak AChE interactors.

None of the fusion constructs activated the second reporter gene - LacZ (see Figure 18). The fusion constructs were transformed into the L40 strain either alone or together with a vector that contains the AD of Gal4 (pGADGH). In this manner, it was tested if the construct alone is activating the reporter genes, or if the construct it is somehow binding to the Gal4-AD and through this binding activates the LacZ gene. A pair of two known interactors, RalV23 and RLIP, served as positive control of the assay (Jullien-Flores *et al.*, 1995). The negative control was the transformation with the empty yeast two hybrid vectors pVJL11 and pGADGH. When transformed in yeast, the two vectors express independently the LexA BD and the Gal4 AD, but the two domains of the transcription factor fail to find each other in order to activate the reporter genes.

The longest construct (pVJL11-N583A) was chosen to be used for the screen. However, the shorter constructs also did not have trans-activating properties (see Figure 18B and C) and therefore were used later in the study to define the interaction site on AChE.

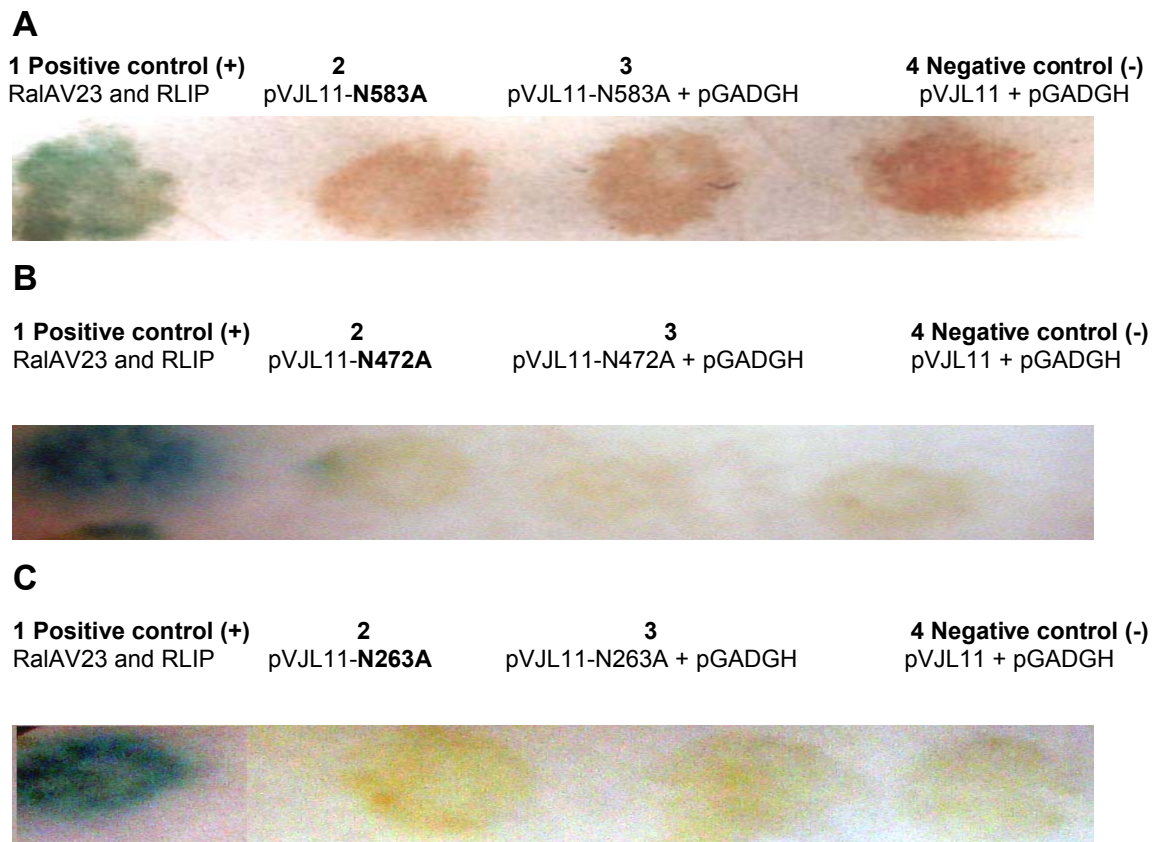


Figure 18. Test for the activation of the LacZ reporter gene of the constructed baits.

The figure shows the results of X-Gal filter lift assay with yeast harboring different plasmids. **(A)**, **(B)**, **(C)** Lanes 1 show the positive control, RaLV23 and RLIP, a pair of two interacting proteins. The blue color is given by the hydrolysis of the X-Gal substrate by the β -galactosidase (the LacZ gene product). **(A)**, **(B)**, **(C)** Lanes 4 show the negative control of the assay, yeast cells transformed with the pVJL11 vector (encodes for LexA BD) and the pGADGH vector (encodes Gal4 AD). **(A)**, **(B)**, **(C)** lane 2 presents yeast colonies transformed with the constructed baits pVJL11-**N583A**, pVJL11-**N472A**, and pVJL11-**N263A**, respectively, and tested for the activation of the LacZ gene. **(A)**, **(B)**, **(C)** lanes 3 shows yeast colonies transformed with pairs of pGADGH and pVJL11-**N583A**, pVJL11-**N472A**, or pVJL11-**N263A**, respectively. No activation of the LacZ gene was observed for any of the 3 bait constructs.

3.2.1.3. Control of expression of the hybrid proteins in yeast

To verify that the absence of auto-activating properties was not due to the lack of cDNA or fusion protein, the presence of cDNA and the expression of the fusion protein inside yeast were tested by colony PCR and Western Blot, respectively (see Figure 19).

PCR done with yeast colonies transformed with pVJL11-N583A, using the mouse AChE specific primers EcoRI AChE – Pst I 2060 and EcoRI AChE - Sph I 1790, respectively, showed the presence of a 1040 bp and 678 bp product (see Figure 19A). The PCR products had the expected size, confirming that the yeast was transformed with the pVJL11-N583A. Western Blot was made with protein extracts from the L40 yeast transformed with pVJL11-N583A. Western analysis showed that the goat polyclonal antibody anti mouse AChE (raised against a peptide mapping at the N-terminus of mouse AChE) recognized a ~ 75 kDa protein (see Figure 19B). This was a bit higher than the predicted molecular weight. However, the hybrid protein might be migrating more slowly, and therefore have a higher apparent molecular weight in SDS-PAGE gels. The antibody did not recognize the shorter construct pVJL11-N472A (not shown). This might be due to the incorrect folding of the protein or a masking of the epitope.

PCR and Western Blot showed that the AChE DNA and the protein are present in yeast. Therefore, the pVJL11-N583A construct can be used to screen a cDNA library for AChE interaction partners.

3.2.2. Two-hybrid screen for AChE interaction partners

3.2.2.1. Characterization of the MATCHMAKER mouse brain cDNA library

A general rule for choosing a library is to select one prepared from a tissue in which the target protein (here AChE) is known to be biologically relevant. In this case an adult mouse brain library was chosen (Clontech). The cDNA library was cloned in the pACT2 vector, which contains the Gal4 activating domain (GenBank accession no: U29899) into XhoI/ EcoRI cloning sites. The number of library independent clones was 3.5×10^6 , the average cDNA size was 2.0 kb, and the estimated % of colonies with inserts was 95%. The number of colonies with inserts given by the producer was tested by randomly picking 100 colonies, isolating the plasmid DNA and doing a restriction analysis. 62% of colonies had an insert. The library titer was determined by plating different library dilutions on LB-Amp plates, counting the colonies and using the producer's formulas. The calculated titer was 0.335×10^9 cfu/ml (cfu- colony forming units).

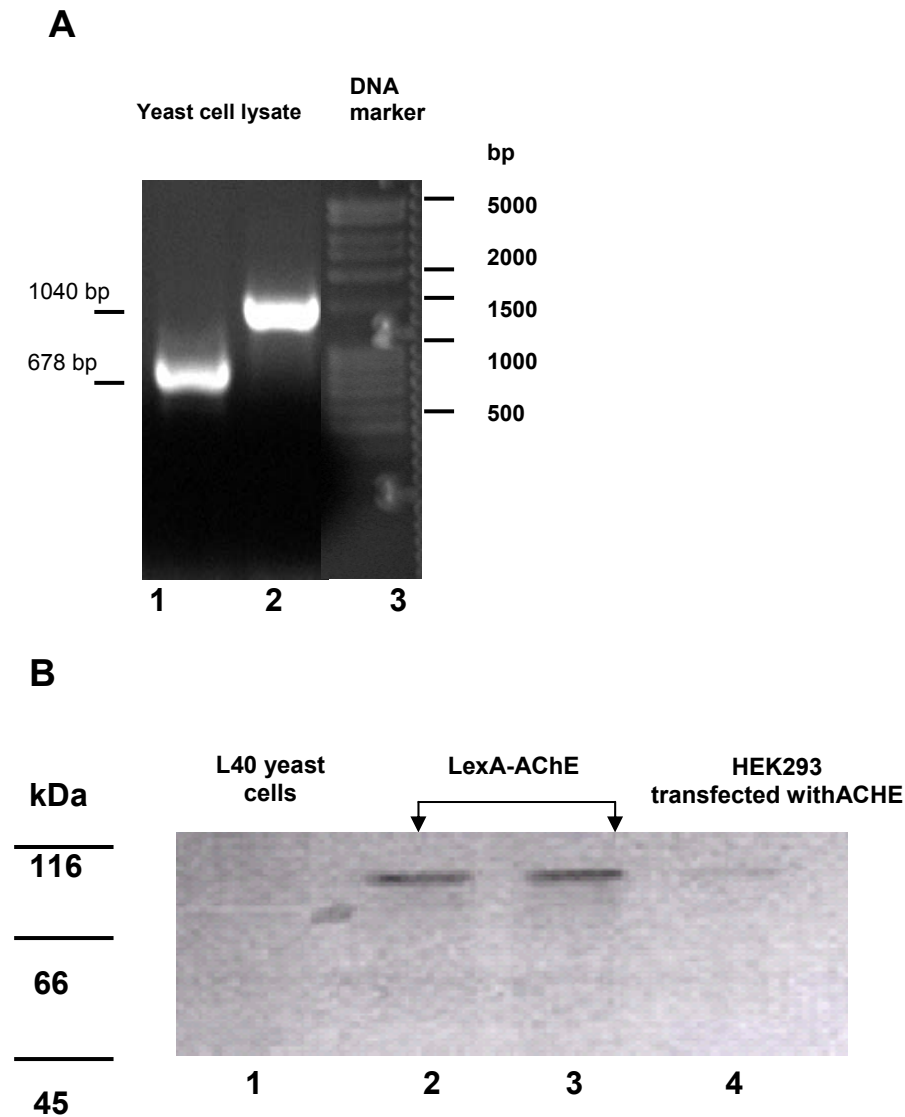


Figure 19. Confirmation of the DNA presence and protein expression in the transformed yeast. (A) 10 μ l of the PCR reaction were separated on a 1% agarose gel and visualized with ethidium bromide under UV. Lane 1 and 2 show the presence of a specific PCR product with the calculated size. (B) Western blot analysis of yeast cell protein extracts. Proteins were separated with SDS-PAGE on a 7.5% gel and transferred to nitrocellulose membrane prior to immunodetection with an anti-mouse AChE antibody. Lane 1 is the negative control, a cell protein extract from yeast not transformed with the pVJL11-N583A. Lane 4 shows the positive control, a \sim 75 kDa protein (68 kDa is the un-glycosylated form), present in the HEK 293 cell lysate (transfected with an expression vector encoding for AChE). Lanes 2 and 3 show a protein that has about the same molecular weight as the positive control (estimated MW was about 61kDa for the AChE plus the Lex A fragment).

3.2.2.2. Screening for interaction partners of AChE - Library transformation

All the tests (auto-transactivation and protein expression) showed that the fusion LexA-AChE could be employed as bait in a two hybrid screen using the L40 yeast strain (see 3.2.1.2. and 3.2.1.3.). However, the L40 showed a basal expression of *HIS3* reporter gene. This expression was inhibited by adding 2.5 mM 3-AT in the yeast growth medium. The bait AChE and the library were sequentially transformed into the yeast, which means the cDNA library was transformed into the L40 yeast strain that already hosts the bait plasmid pVJL11-N583A. The idea of a successful screen is to saturate the screening to obtain as many transformants as possible, and to avoid transformation of a single yeast cell with multiple library plasmids, which can seriously complicate subsequent analysis. The employment of the sequential transformation gives better results for such a screen. Two independent library transformations were made, using the LiAc method (see 2.2.2.4.). This method typically results in transformation efficiencies of 10^6 transformants per mg of DNA when using a single type of plasmid. Approx. 2×10^6 transformants were screened in these 2 experiments. The transformants were plated out on DO/-Leu/-Trp/-His plates that are selective for the two plasmids and the additional auxotrophic reporter gene *HIS3*. The plates included also 2.5 mM 3-AT. After 5-6 days growth at 30°C the first colonies were picked. The size of the colony was considered to reflect the strength of the interaction, the bigger the colony is, the stronger is the interaction. However, the small colonies should not be considered background. Due to the high number of colonies that grew, just 190 big and middle size colonies were further analyzed. The transactivation of the second reporter gene LacZ was tested by analyzing β -galactosidase activity using the β -galactosidase filter assay described in 2.2.2.5. Most of the positive clones chosen to be further analyzed turned blue within 5 hours at 30°C.

3.2.2.3. Checking the potential positive clones - insert analysis, assay of the specificity in the yeast two-hybrid system

A large fraction of the 190 His⁺ colonies showed no blue color with the β -galactosidase filter assay, and therefore they were not further analyzed. 96 colonies displayed β -galactosidase activity (see Figure 20). In order to control the presence and size of the library cDNA, yeast colony PCR with pACT2 specific primers was performed (see 2.2.1.10. for protocol). Due to the big size of the library inserts, these PCRs were not yielding to the complete amplification of the library cDNA. Therefore, the plasmid DNA was isolated from all 96 *HIS*⁺/*LacZ*⁺ colonies (see 2.2.2.7.) and electroporated into *E. coli* strain

HB101. These yeast minipreps contained both the bait and the library hybrid plasmid. Thus, the HB101 strain was used. HB101 had a defect in the *leuB* gene, which could be complemented by the *LEU2* gene from yeast vectors. These bacteria, when grown on M9 selective medium, selected just for the library plasmids (Leu^+). Library plasmid was subsequently isolated from yeast and analyzed by restriction enzyme digestion. This digestion showed that a small part of library plasmids had no insert. Figure 20 gives an overview of the screen and shows the results of the intermediary steps.

2 million yeast transformants screened



190 His^+



96 $\text{His}^+/\text{LacZ}^+$



60 had an insert



23 pVJL11-N583A-dependent $\text{His}^+/\text{LacZ}^+$

Figure 20. Summary of the yeast two-hybrid screen for AChE interacting proteins.

Yeast strain L40 was transformed with pVJL11-N583A and selected for Trp prototrophy. These yeast cells were transformed with a mouse brain cDNA library cloned into the pACT2 vector. Yeast cotransformants containing putative AChE interacting partners were selected by growth on minimal medium lacking Trp, Leu and His. The His^+ clones were tested for the β -galactosidase activity and 96 showed activity. 60 of the library fusions were shown to contain a cDNA library insert. 23 of the library plasmids were found to produce β -galactosidase activity only when cotransformed with pVJL11-N583A, but not when cotransformed with the control fusion plasmids pVJL11-LexA-Lamin and pVJL11-LexA-p53.

Because many clones obtained from the yeast two-hybrid screen are false positives, it is essential to test the interaction of the individual pACT2-Gal4-cDNA clones with the pVJL11-N583A and with an unrelated protein such as lamin and p53. Therefore, before sequencing, all the library fusions were retransformed in yeast in the following combinations:

- alone, in order to see whether they have autoactivating properties,
- with the empty vector pVJL11,
- together with the pVJL11-N583A (to re-test the interaction found by screening),
- and together with pVJL11-lamin or pVJL11-p53.

Figure 21 shows an overview of the β -galactosidase activity assay for all the yeast two-hybrid positives.

3.2.2.4. Prey overview

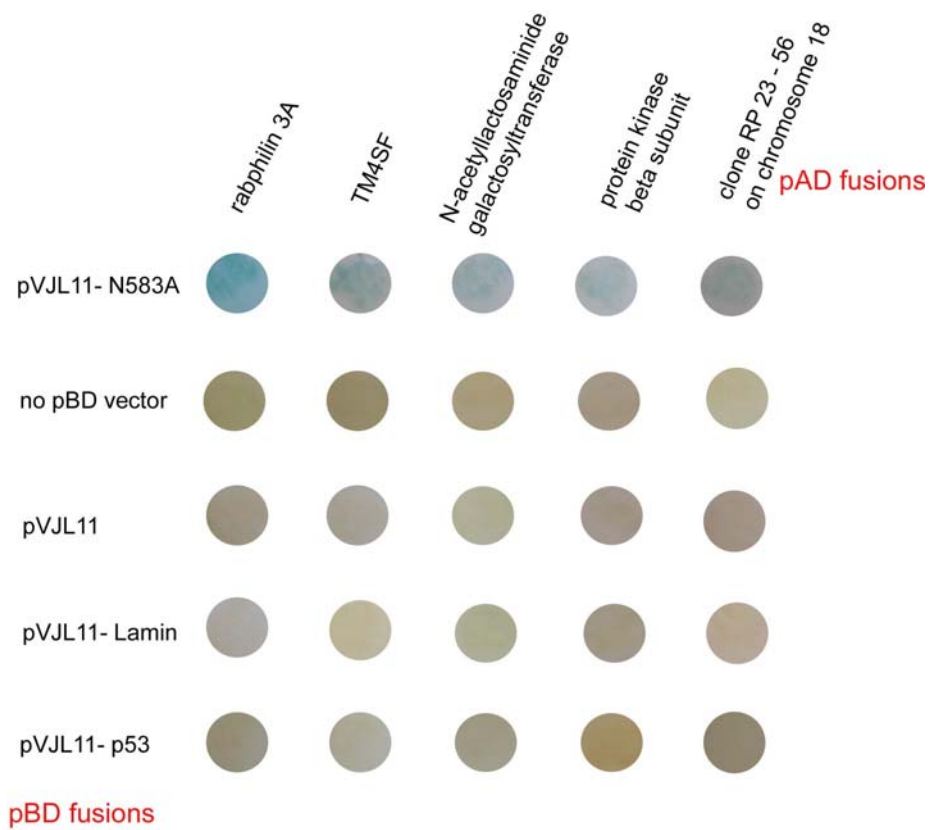
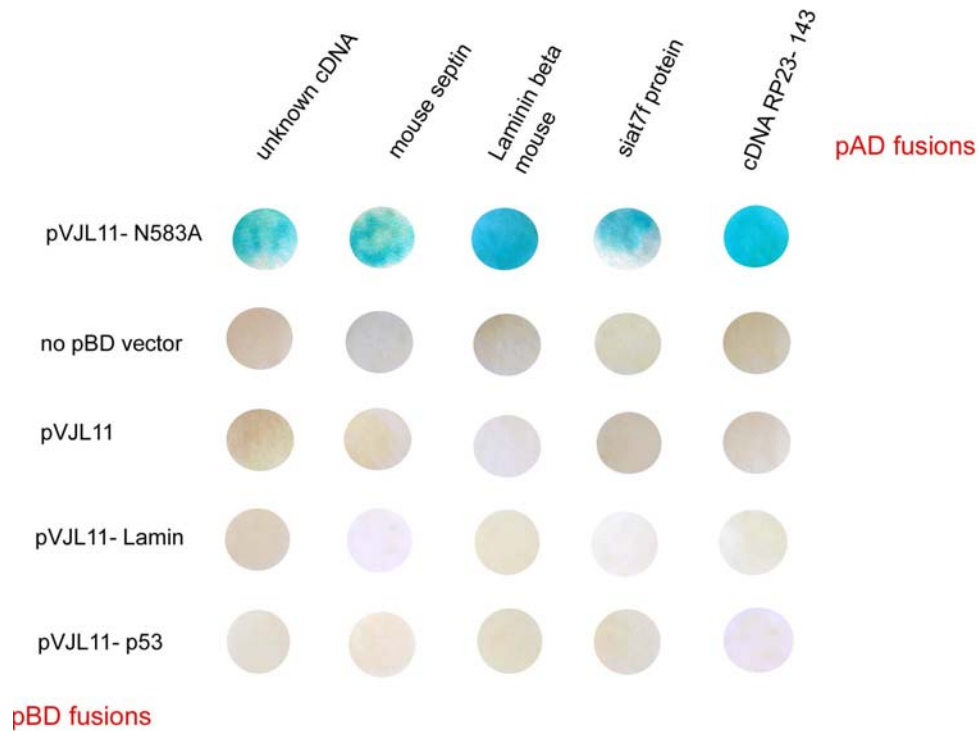
Much more information about the prey was derived from sequence data. The identity of the AChE interacting proteins was determined by sequencing directly from the pACT2 vector using standard automated DNA sequencing methods. The derived cDNA sequences were analyzed using *blastx*. A search for homologues proteins in all the reading frames was done. The results are presented in Table 1. The reading frame of the sequenced DNA was compared with the ORF of the pACT2 vector. The listed names of the clones (Table 1) are in conformity with the pACT2 ORF and represent the smallest *blastx* eValue result. By analyzing the obtained sequences, it was possible to classify inserts into two main groups; those of potential interest, and the others. Some proteins can also be excluded from the list of interesting interactors if they are part of a list of known false positives that are frequently found, independently of the target used (e.g. Zn-finger proteins, hsp, ribosomal proteins).

In conclusion, screening a brain cDNA library for AChE-interaction partners proved to be a successful method. At the end of the control procedures 23 clones were identified as 'true' positives. The proteins encoded by these clones are binding partners of acetylcholinesterase in yeast.

(next page) Table 1. Overview of the AChE potential binding partners. β -Galactosidase activity was determined by filter lift assay for the L40 strain cotransformed with the indicated plasmids. +++, ++, + represent colonies that turned strongly blue, moderately blue, and weakly blue, respectively; - represent colonies that did not turn blue after 5 hours incubation. The homologues represent the *blastx* search that gave the smallest eValue. The clone numbers with the same colour represent fragments that are either identical, or belonging to the same protein.

Clone #	Growth on DO/- His	LacZ Test	LacZ test after the retransformation in combination with					Homologies
			-	pVJL11	pVJL11-AChE	pVJL11-Lamin	pVJL11-p53	
8	+	+	-	-	+	-	-	unknown mouse cDNA, no homologies
10	+	+	-	-	++	-	-	mouse septin
12	+	+++	-	-	+	-	-	mouse laminin beta 1
13	+	+	-	-	+	-	-	Siat7f protein, sialyltransferase
20	++	+	-	-	+	-	-	mouse DNA from clone RP 23 143
23	++	+	-	-	+	-	-	rabphilin 3A
24	+	+	-	-	++	-	-	unknown cDNA
29	+	+++	-	-	+	-	-	N-acetyllactosaminide alpha-1,3-galactosyltransferase
33	+	++	-	-	+	-	-	mouse DNA from clone RP 23 143
43	+	+	-	-	+	-	-	Protein kinase beta subunit
47	+	+	-	-	+	-	-	mouse DNA clone RP 23 - 56 on chromosome 18
50	++	+	-	-	+	-	-	mouse DNA from clone RP 23 415c3
55	+	+	-	-	+	-	-	mouse DNA from clone RP 23 143
56	+++	++	-	-	++	-	-	mouse DNA from clone RP 23 354 K2
58	+	+	-	-	+	-	-	mouse DNA from clone RP 23/actin gamma cytoplasmic
60	+	+	-	-	+	-	-	mouse Tbk1
62	++	++	-	-	+	-	-	adult male cerebellum cDNA, hypothetical Zn-finger CChC type containing protein
63	+	+	-	-	+	-	-	mouse DNA clone RP 23 - 56 on chromosome 18
70	+	+	-	-	+	-	-	mouse embryo 12 days DNA
78	+	++	-	-	+	-	-	mouse creatine kinase
88	++	+	-	-	+	-	-	mouse, similar to HBx Ag transactivated protein 2
91	++	+	-	-	++	-	-	mouse creatine kinase
96	+	+	-	-	++	-	-	mouse Tbk 1

β -Galactosidase filter lift assay with the „putative“ AChE-interaction partners, part 1



β -Galactosidase filter lift assay with the „putative“ AChE-interaction partners, part 2

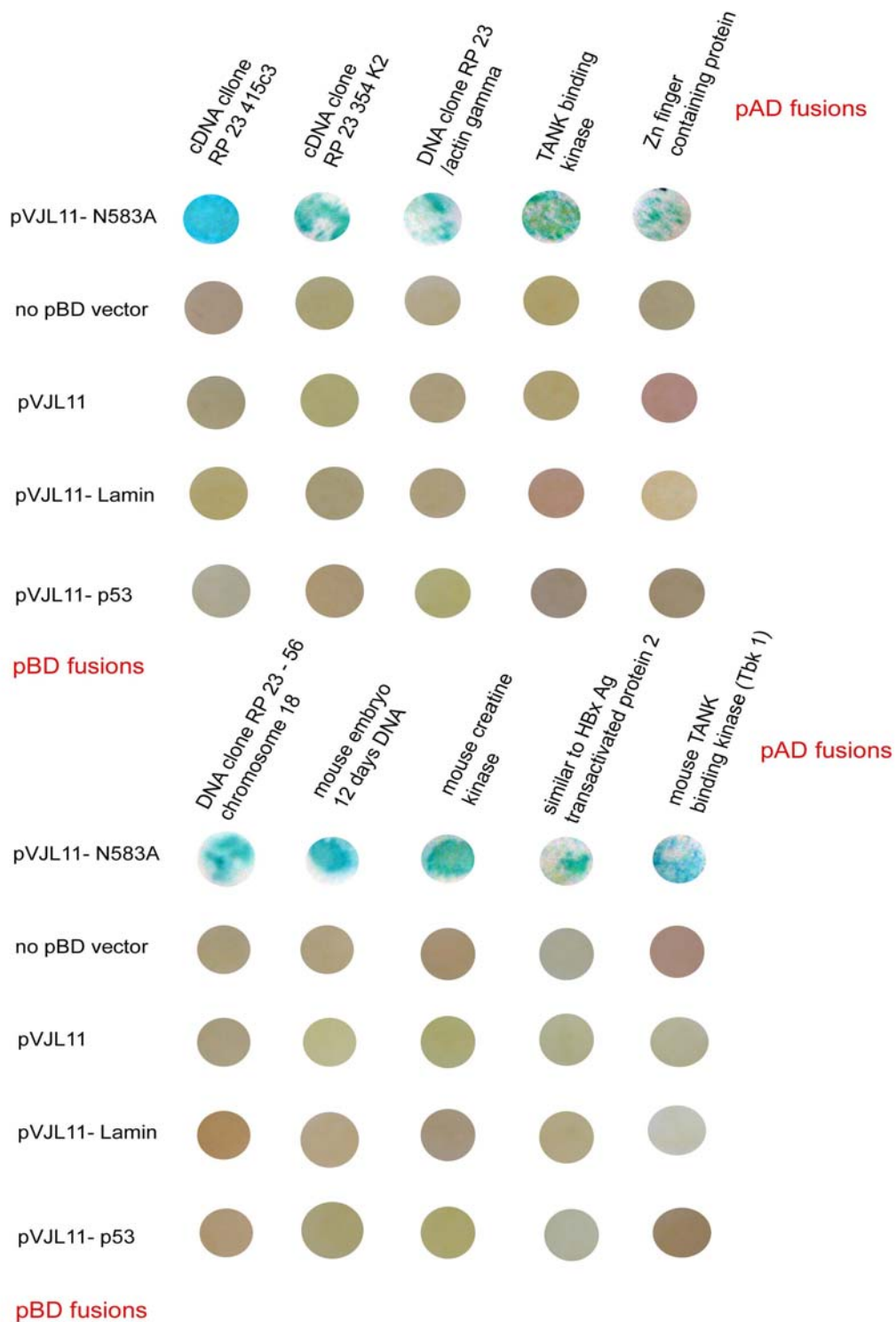


Figure 21. β -Galactosidase filter lift assay with the „putative“ AChE interaction partners. Shown are L40 yeast cells that were transformed with different combinations of AD (Gal4) and BD (LexA) fusion proteins. An interaction between a fusion protein pair will give a blue color of the filter. The protein pairs that do not interact are, depending on the color of the yeast colony orange-yellow, pink or white.

PART III: AChE interacts with laminin-1 β

3.3.1. AChE – laminin-1 interaction in the yeast two-hybrid system

A laminin fragment of 898 bp (see Figure 23) was found as interacting with AChE in a yeast two-hybrid screen using as bait the full length core AChE (583 aa). After sequencing the clone, computational analysis was done with NIH-*blastn* and *blastx* (from PubMed website), see Table 2. By screening 2×10^6 clones, the laminin-1 β chain appeared once as AChE putative-interaction partner. To confirm the interaction, the laminin fragment was re-transformed in yeast together with the pVJL11-N583A and tested for the activation of the LacZ reporter gene (Figure 22). To determine the possible binding site of AChE on laminin-1, two shorter LexA-AChE constructs were transformed together with pACT2-Laminin into L40 yeast strain. Figure 22 shows that the intensity of the signal in the β -galactosidase test decreased with the length of the construct. The shorter construct, pVJL11-N263A, activated weakly the LacZ gene.

The data indicated that in the view of the results of the β -galactosidase filter lift assay, the interaction between AChE and laminin-1 is moderately strong and that the region containing 240th aa residue to 503th aa residues of AChE is essential for interaction with laminin-1 β .

Blastx result	eValue	Identity
laminin B1 subunit 1 [Mus musculus]	e-178	98%
Laminin beta-1 chain precursor (Laminin B1 chain)	e-178	98%
similar to laminin B1 [Rattus norvegicus]	e-175	98%
laminin, beta 1 precursor [Homo sapiens]	e-168	92%
laminin, beta 1 [Danio rerio]	e-128	72%

Table 2. The results of the *blastx* alignments for the clone 12.

G C R P C D C D L G G A L N N S C S E D S G
*ctcgagatggg*tgtcgtaccttgtgactgtgaccttggagggcgctgaacaatagctgctccgaggactccggc
Q C S C L P P M I G R Q C N E V E S G Y Y F T T L
agtgtcctgctgcccccatgattgggcggcagtgtaacgaggtggagtcgggttactacttcaccaccctg
C D H Y I Y E A E E A N L G P G V V V V E R Q Y I
gaccactacatctacgaagccgaggaagccaatctggggcctggagtcggttgtggtggaaaggcagtacattcag
Q D R I P S W T G P G F V R V P E G A Y L E F F I
gaccgcatttccttctggacaggacctggcttcgctccgggtgcctgaaggggcttatttggagtttttcattgac
D N I P Y S M E Y E I L I R Y E P Q L P D H W E K
aacataccatattccatggagtatgaaatcctgattcgctatgagccacagctgccggaccactgggagaaagct
A V I T V Q R P G K I P A S S R C G N T V P D D D
gtcatcactgtacagcggccggggaagattccagccagcagccgatgtggtaacaccgttccccgatgatgacagc
S Q V V S L S P G S R Y V V L P R P V C F E K G M
caggtggtgtccttgtcaccggggtcaagatacgttgtcctccctcgccccgtgtgctttgagaagggaatgaac
N Y T V R L E L P Q Y T A S G S D V E S P Y T F I
tacacggtgaggttggagctgccccagtatcacggcatcgggcagtgacgtggagagcccttacacgttcatcgac
D S L V L M P Y C K S L D I F T V G G S G D G E V
tcgcttgttctcatgccctactgtaaatcgctggacatcttcactgttggcgggtcaggcgatggggagggtcacc
T N S A W E T F Q R Y R C L E N S R S V V K T P M
aatagtgcctgggaaaccttccagcgctacaggtgtctggagaacagcaggagtggtggtaaaaacacccatgaca
T D V C R N I I F S I S A L I H Q T G L A C E C D
gatgtctgcagaaacattatcttcagcatttctgccttgattcaccagacgggccttgcttgtgaatgtgacccc
P Q G S L S S V C D P N G G Q C Q C R P N V V G R
cagggatctctgagttctgtgtgtgacccccaatggaggccagtgccagtgccgtcctaattgtggttggagaacc
T X N
tncaaca

Figure 23. Nucleotide and amino acid sequence of clone 12, corresponding to mouse laminin-1 β . The open reading frame is +3. The sequence starts with the XhoI restriction site.

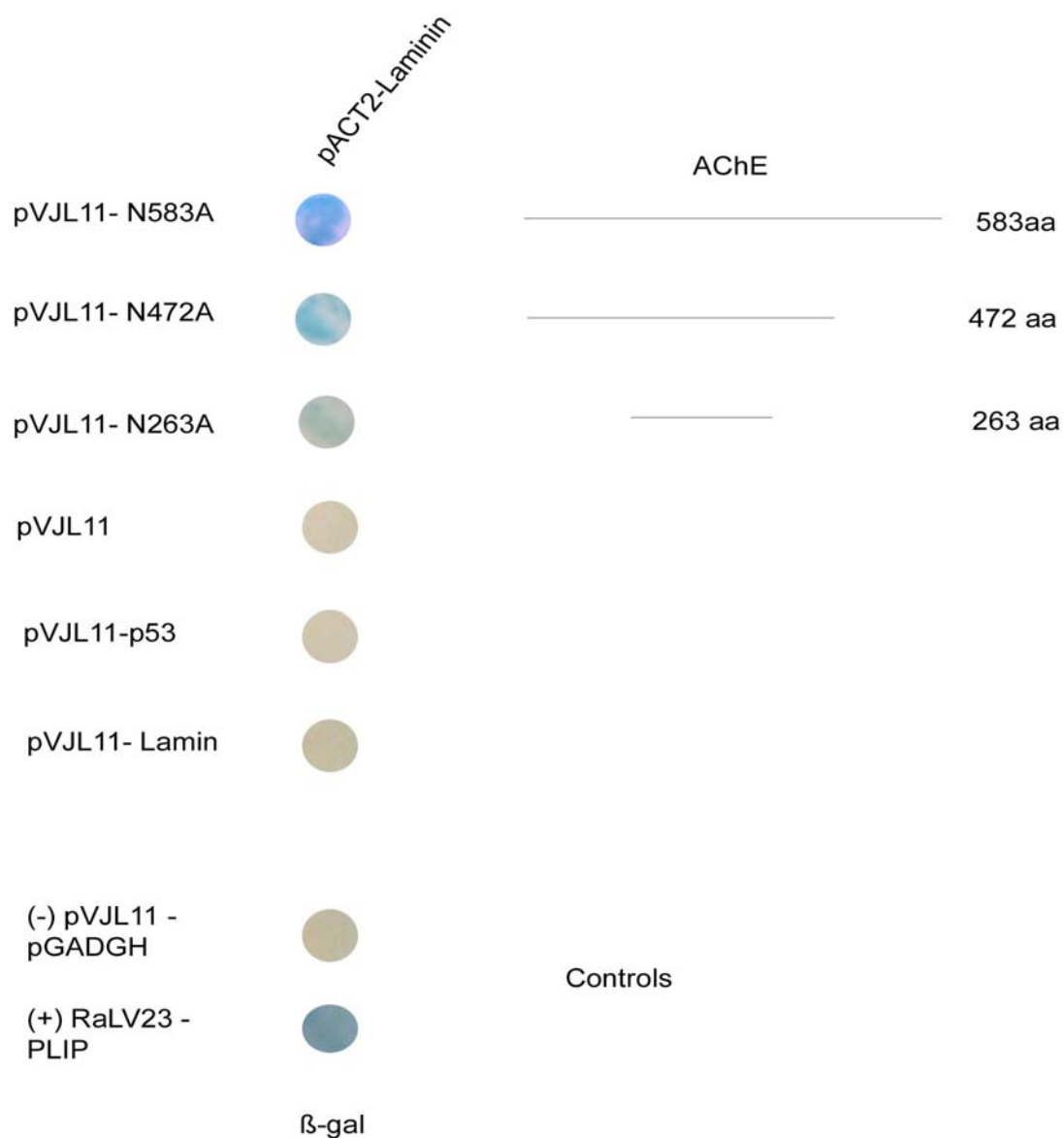


Figure 22. Interaction of AChE constructs with laminin-1beta on L40 yeast. Shown are the β -galactosidase activity test results. Blue: interaction, white: no interaction. On the right side there is a schematic alignment of the AChE constructs used.

3.3.2. AChE interacts with laminin 1 – confirming the interaction through coimmunoprecipitation experiments

After finding that laminin-1 binds to AChE in the yeast two-hybrid system, it was important to see a confirmation of the interaction outside the yeast cell. To such end, several assays are available, such as *in vitro* “pull-down” assays and coimmunoprecipitation. As already mentioned, AChE is a glycoprotein and has intrachain disulfide bridges. A pull-down assay was from the beginning not suitable, because the system is based on expression of fusion-proteins in bacteria. Data until up to now showed that in bacteria AChE will be expressed in an inactive form and accumulated in inclusion bodies (see Heim *et al.*, 1998). Therefore, a coimmunoprecipitation assay was chosen.

2 different approaches were made (see also Figure 24):

- by using an anti-mouse AChE (goat polyclonal) or an anti-mouse Laminin (rabbit polyclonal, rat monoclonal) antibody, to coimmunoprecipitate laminin, respectively AChE from *mouse brain membrane protein* extracts;
- AChE was *overexpressed* in the HEK293 cell line by transfection with pCDNA3-AChE, and laminin-1 was added as protein (purchased from Sigma) into the *cell lysate*. The same antibodies mentioned above were used.

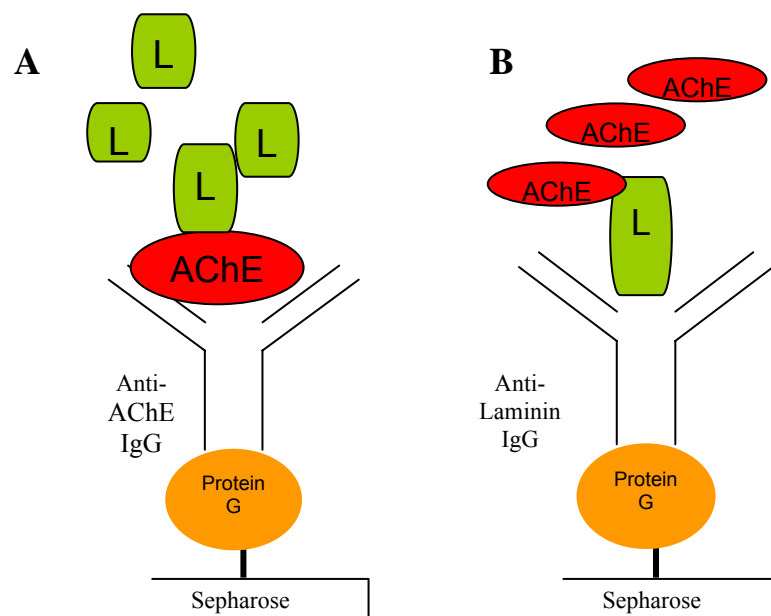


Figure 24. Experimental design for the coimmunoprecipitation of AChE and Laminin.

(A) An antibody against N-terminus of AChE was used to co-immunoprecipitate laminin-1 from a brain extract or cell lysate. The antibody-AChE-laminin-1 complex was isolated with the help of Protein G-Sepharose. (B) An antibody against laminin-1 was used to co-immunoprecipitate AChE. Protein G-Sepharose was used to isolate the antibody-laminin-1-AChE complexes. L- Laminin.

From the 3 antibodies available, 2 were shown to immunoprecipitate the protein: the goat polyclonal anti-AChE could precipitate AChE from a brain extract (Figure 25A), and the rabbit polyclonal anti-laminin-1 could precipitate laminin-1 (Figure 25A, lane 3). The rat monoclonal anti-laminin did not seem to bind to laminin-1 and was not further used (Figure 25A, lane 2). HEK293 cells were transfected with an AChE expression vector (pCDNA3-AChE). AChE activity was measured in both brain extracts (~ 4.6 mU/ mg protein) and HEK 293 cell lysates (~5.5 mU/mg). The first attempts to coimmunoprecipitate AChE and laminin from the brain extracts were not successful. This may be also due to the high salt – detergent buffer used to solubilize the membrane proteins (1 M NaCl, 0.5% Triton X-100). The buffer was then changed by dialysis with PBS, 0.1% Triton X-100. After dialysis the AChE activity decreased generally by 1 mU.

Figure 25B and 25C shows that using the goat polyclonal anti-AChE laminin-1 was coimmunoprecipitated with AChE, both from brain extract or HEK293 cell lysate, when these extracts were “spiced” with 5 µg laminin-1 (Sigma). The rabbit polyclonal anti-laminin was not able to coimmunoprecipitate AChE and laminin. The results of the Coomassie staining showed a band corresponding to the ~70 kDa AChE (Figure 25B, lane 3), but Western analysis revealed no AChE signal. Also, the binding seemed to be dependent on buffer salt concentration. From the 4 different coimmunoprecipitation buffers, PBS with 0.1% Triton X-100 gave the best results. No binding was observed in buffers containing 500 mM NaCl, 250 mM NaCl and 200 mM NaCl.

The summarized results are the following:

- The anti-mouse AChE could precipitate together with AChE (from brain extracts or HEK293 transfected cell extracts) laminin-1, when the amount of laminin-1 in the extracts was increased by adding 5 µg purified laminin-1.
- The anti-mouse laminin-1 gave contradictory results. The antibody could precipitate laminin-1 and also AChE from the HEK293 cell lysates (Coomassie gel staining showed a band corresponding to the AChE MW). No AChE band was present in the Western Blot experiment, making these results not consistent.
- The AChE – laminin-1 binding is inhibited by high salt concentration in the buffer (from 500 mM NaCl to 200 mM NaCl). The best results were obtained by using as binding buffer PBS + 0.1% Triton X-100.

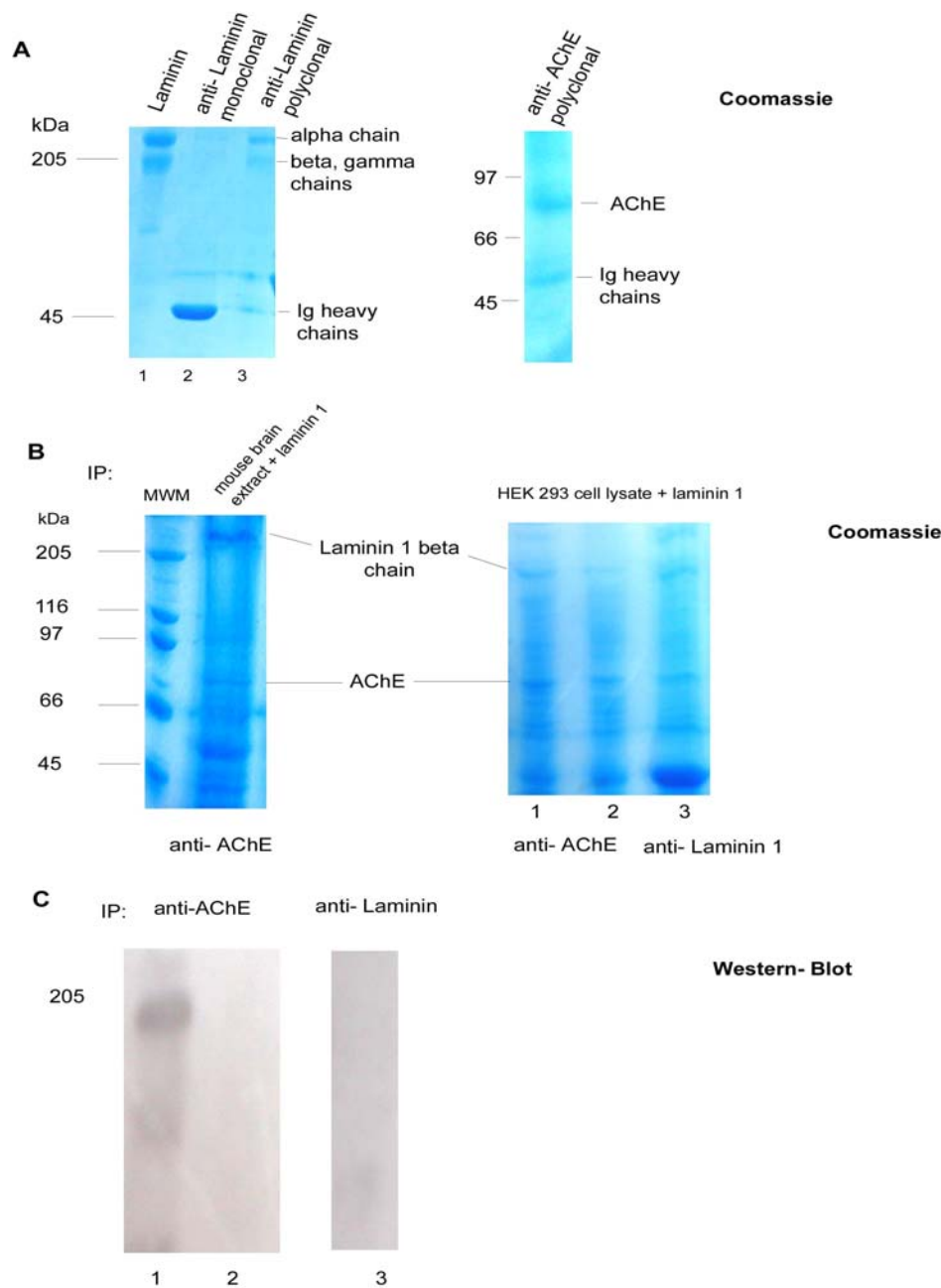


Figure 25. Laminin-1 binds to AChE in vivo – coimmunoprecipitation experiments. (A) shows Coomassie stained 7.5% SDS-PAGE gels. Laminin (lane 1) appears as 2 bands on the gel, a 400 kDa band (the alpha 1 chain) and a 200 kDa band (beta and gamma chains). A monoclonal anti-laminin-1 antibody didn't precipitate laminin 1 (lane 2). A polyclonal anti-laminin-1 antibody could immunoprecipitate laminin-1 (lane3). The polyclonal anti-AChE antibody bound to the AChE (A), right. (B) After immunoprecipitation with an anti-AChE or anti-laminin antibody the proteins were separated on a 7.5% gel and the gel was stained with Coomassie. Using an anti-AChE antibody both AChE and laminin-1 were immunoprecipitated from brain extracts (B) left or from HEK293 cell lysates (B) right lane 1 and 2, respectively. The 200 kDa band corresponds to the laminin-1beta and gamma chains. (C) Western analysis with an anti-laminin antibody of the AChE coimmunoprecipitated proteins.

3.3.3. Co-localization of AChE and laminin-1 in the central nervous system

To determine whether AChE is localized in the same region as laminin-1 in the CNS, immunofluorescence microscopy and Karnovsky-Roots histochemistry studies were done. 20 µm thick adult mouse brains sections were stained with a mixture of anti-mouse AChE and anti-mouse laminin-1 antibodies (see 2.1.16.) and analyzed under a fluorescence microscope. The brain sections were from adult mice 68 to 75 days old. Controls without the first antibody were carried out to ensure that the staining was specific.

The fluorescence microscopy image of an adult mouse brain section reveals laminin-1 staining throughout the nervous system. The stain appears to be confined to the blood vessels (see Figure 26A). Karnovsky-Roots cholinesterase activity staining shows that the CNS stained densely for AChE (Figure 26C) even if here the cholinergic innervation is limited. Figure 26B shows that the AChE can be found also concentrated in nuclei of activity. The antibody staining revealed that AChE was predominantly associated with the nerve cell bodies (Figure 26D), but also on the neurites (Figure 26G). As illustrated in Figure 26F, laminin-1 was mostly present in blood capillaries or at the contact of capillaries with the glia cells. The presence of laminin-1 (EHS laminin; alpha1-beta1-gamma1) and AChE was examined in the CNS. The anatomy and morphology of AChE stained neurons has been examined extensively (Bolam *et al.*, 1984; Ostergaard *et al.*, 1992). The pattern of labeling was consistent with AChE localization described in the stereotaxic atlas and elsewhere using the AChE histochemical method. Laminin-1 is associated with the blood vessels. However, studies of the distribution of the laminin chains showed a different pattern. For instance, literature data reveal that immunostaining for gamma1 chain is present throughout the central nervous system (CNS) in essentially all neuronal cell bodies and their most proximal processes (Hagg *et al.*, 1997).

The results presented in Figure 26 together with the literature data show that AChE and laminin-1 are both expressed in brain and therefore provide support for a direct interaction of the two proteins.

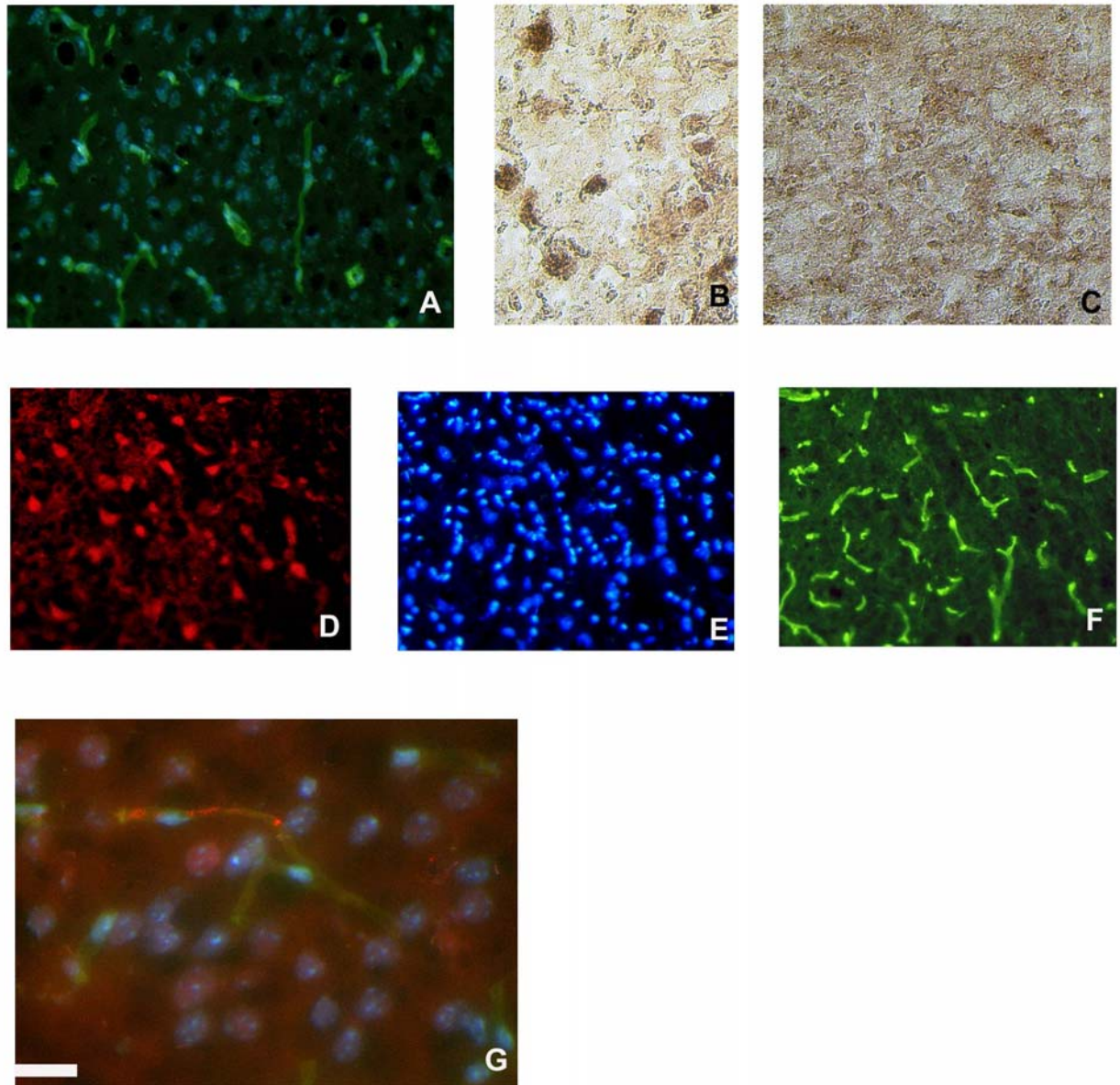


Figure 26. Localization of AChE and laminin-1 in the mouse CNS. (A) Laminin-1 and DAPI staining on a mouse brain section. (B) and (C) represent Karnosky-Roots AChE activity staining. (D), (E), (F) represent the same brain section stained with an anti-AChE (D, red) and an anti-laminin-1 (F, green) antibody. (E) – DAPI staining of cell nuclei. (G) Double staining for AChE (red) and laminin-1 (green). Scale bar 100 μm .

4. Discussion

4.1. Neurexin-1 β expression pattern overlaps with the AChE expression, but neurexin-1 β does not bind to AChE

In the first part of this study, I attempted to show that AChE competes with the homologous neuroligin to bind neurexin-1 β .

4.1.1. AChE and Neurexin-1 β expression during retinal differentiation and synaptogenesis

Neurexin-1 β

Within the vertebrate retina, non-catalytic neurogenic roles have been attributed to AChE based on the spatiotemporal pattern of its expression during development. The presence of the cholinesterase-like domains in the noncatalytic, cell adhesion proteins, e.g. neuroligin-1, may reflect the capacity of AChE for protein-protein interactions. Therefore, we addressed the question whether AChE competes with the cell adhesion molecule neuroligin-1 for the binding to its ligand neurexin-1 β .

Before answering this functional question, the expression of neurexin-1 β was analyzed in the developing chicken retina with the help of *in situ* hybridization and compared with the AChE expression pattern. The staining appeared to be specific, as the hybridization with a control 'sense' probe gave no signal. It was shown that both neurexin-1 β and AChE, two genes whose protein products are involved in neurotransmission, became detectable in differentiating chicken retinal cells much before the synaptogenesis. Indeed, their spatial expression patterns overlapped. Temporally, AChE expression (appearing first at E4) shortly precedes the neurexin-1 β (first seen at E6). The neurexin-1 β gene was first detected in the ganglion cell layer, followed by the expression in the inner half of the inner nuclear layer. This pattern remained constant during retinal development and was limited to the above mentioned layers. In the retina, ganglion cells are generally considered to be generated first; therefore it was predictable that the first neurexin-1 β transcripts would be present in the ganglion cells. The first embryonic day where the neurexin-1 gene appeared was E6, corresponding to the stage of intense ganglion cell differentiation. The neurexin expression pattern superimposes the steps of retinal differentiation toward synaptogenesis.

The first neurexin signals appeared in the ganglion cell layer at the E6 when a vast majority of retinal cells are still proliferating, except for a part of ganglion cells that start to differentiate. At E9 when the retina is largely post-mitotic, but most of the cells remain morphologically undifferentiated, a specific pattern of expression became obvious and remained qualitatively unchanged at E13 and E19. During all retinal development the photoreceptors did not express neurexin-1 β .

Up to now, there are no published studies about the neurexin-1 β expression in the retina. However, the results were not surprising considering the roles of neurexins. These proteins function as heterophilic cell adhesion molecules (Nguyen and Südhof, 1997) and can also affect the fusion of neurotransmitter vesicles with the plasma membrane (Sugita *et al.*, 1999). The regulation of vesicle fusion, as well as cell adhesion may affect the progression of growth cones toward their targets as well as the transition from neurite extension to synaptogenesis. This neurexin expression pattern was comparable with the neurexin expression in developing mouse brain (Puschel and Betz, 1995). Transcripts of neurexins were detected in the mouse brain starting at E10 and increased with the maturation of the nervous system. RNAs of neurexins were found throughout the nervous system exclusively in postmitotic neurons (similar to the chick retina) and at least one day before synapses are formed. To date, this is the first study to report neurexin-1 β localization in the retina.

AChE

AChE is considered a marker for differentiating retinal cells (Layer and Willbold, 1995). When AChE was overexpressed in mice, an embryonic feedback mechanism drastically lowered the neurexin-1 β transcripts in spinal cord neurons. This suggested that embryonic neurexin expression is concerted *in vivo* with the AChE levels (Andres *et al.*, 1997). To confirm this, AChE staining in the developing retina was undertaken. Similar to previous studies of this type (Layer *et al.*, 1987), the staining confirmed the presence of AChE-positive cells in the ganglion cell layer, in the inner nuclear layer, and in 2 sub-bands in the inner plexiform layer.

In the current study both the histochemical activity staining (Karnovsky-Roots), and the antibody staining with a monoclonal antibody against chicken AChE were used (Tsim *et al.*, 1988). The AChE labeling appeared to be specific, since the omission of the first antibody significantly reduced labeling. When staining for AChE, most research groups use the histochemical method. Typically, histochemical methods can lead to non-specific labeling of all cholinesterases, since butyrylcholinesterase (the other cholinesterase) can

also hydrolyze acetylcholine. Therefore iso-OMPA, a specific inhibitor for the butyrylcholinesterase, was used for the histochemical staining. Antibodies have the advantage that they do not rely on the cholinergic activity of cholinesterases; therefore they can be raised against particular species of AChE and diluted to the required level for cellular labeling. Furthermore, within neurons there is non-activated AChE (Massoulié *et al.*, 1993), which would be revealed by antibody staining but not by the Karnovsky-Roots method. However, AChE-stainings of chicken retina yielded similar results for both methods (enzymatic-light and fluorescent).

The first AChE-positive cells were observed at E4 in the central part of the retina. This correlates with the hypothesis that the expression of AChE indicates one of the earliest postmitotic events in the life of differentiating neurons. Although very few amacrine cells would be cholinergic innervated, it appeared that a large population of cells from the ganglion cell layer and the inner nuclear layer express an active form of AChE. This might be attributed to the non-cholinergic, morphogenic role of the protein during retinal differentiation.

The staining for AChE displayed the presence of the enzyme also in the synaptic inner plexiform layer, where the labeling appears in the form of two sub-layers from E13 onwards. Stratification at the IPL level has been observed from about embryonic day E9. The first band is formed by cholinergic cells at E9 or E10, followed by a second cholinergic sub-band at E13. The retinal inner plexiform layer (IPL) is a highly organized synaptic zone, where visual information is transmitted from bipolar cells onto ganglion cells and is controlled and modified by amacrine cells through laterally interconnecting branches. Cajal (1972) previously established the existence of five sublayers in the vertebrate retina. The layers reflect zones where specific sets of cells interconnect. The development of these sub-layers is not well understood. However, the early presence of AChE-sublayers in the chicken retina, before synaptogenesis, suggests a non-cholinergic, pre-synaptogenetic role for AChE.

From these labeling studies it was possible to conclude that:

1. The spatial expression of neurexin-1 β was correlated with the AChE in the chick retina.
2. Temporally: the first neurexin-1 β transcripts appeared 2 days later compared with AChE.
3. The expression of both markers was intense during the differentiation of the neurons that send their axons and dendrites in the inner plexiform layer to form synapses (ganglion, amacrine and horizontal cells).

This distribution suggests that AChE supports the neurite outgrowth, while neurexin-1 β could be involved in pathfinding and synaptogenesis in the retina. Unfortunately, double stainings were not done for the AChE and neurexin-1 β , so it is impossible to claim that the same cell population was labeled with these two different markers. However, mouse AChE shows no binding to neurexin-1 β at concentrations up to 100-fold above where neuroligin-1 binding to neurexin-1 β can be demonstrated (Comoletti *et al.*, 2003). The absence of AChE binding does not appear to be a consequence of the different glycosylation pattern on AChE, because a glycosidase-treated AChE also shows no detectable neurexin-1 β binding (Comoletti *et al.*, 2003). Nevertheless, as already mentioned, these results do not exclude the possibility that AChE binds to different neurexin isoforms in other experimental systems.

4.1.2. Expression patterns of other retinal markers

The Expression of Synaptotagmin I precedes synaptogenesis and correlates with neurexin-1 β expression

The adhesion function of neurexins is explained by its binding to neuroligin. The second already mentioned function of neurexins, the regulation of neurotransmitter vesicle fusion with the plasma membrane, implies another interaction, which is the binding of neurexin's intracellular domain to synaptotagmin I (Hata *et al.*, 1993). Synaptotagmin, a major intrinsic membrane protein of synaptic vesicles, interacts with the cytoplasmic domain of neurexins; the binding is calcium-independent and of moderate affinity. To confirm that neurexin-labeled neurons are also synaptotagmin-positive, the expression pattern of synaptotagmin I in the chicken retina was analyzed with *in situ* hybridization.

Synaptotagmin I mRNA was expressed in the ganglion cell layer starting with the E6. The early expression of synaptotagmin can not be explained by their classical role as synaptic vesicle proteins, since the first steps of synaptogenesis in the inner plexiform layer take place about E12 (Sheffield and Fishman, 1970). At E9, not just GCL, but also the inner nuclear layer displayed synaptotagmin I signals. This pattern remained constant throughout the retinal development and paralleled the neurexin-1 β expression in the chick retina.

The expression of these two markers in the retina can be correlated with processes of presynaptic differentiation. The presynaptic active zone is created through the assembly of elaborate macromolecular complexes (Garner *et al.*, 2002). These processes can easily take place in small cells, e.g. epithelial cells, but the neuron's morphology creates unique trafficking and sorting problems. One of the ways to solve this problem is the generation of precursor vesicles and the early maturation of synaptic vesicles. Before synaptogenesis, the

non-regulated fusion of these vesicles at non-synaptic sites leads to detectable levels of neurotransmitter along the growth cones (Hannah *et al.*, 1999). The contribution of the released neurotransmitter to the synaptogenesis is unknown. However, this mechanism can explain why synaptotagmin I was expressed immediately after axon outgrowth. The protein could be a resident of heterogeneous organelles that probably represent a mixture of precursors for synaptic vesicles and the axon plasma membrane.

Neurofilament M transcripts are present solely in the retinal ganglion cells

Neurofilaments are nerve cell specific intermediate (10 nm) filaments. Together with actin microfilaments and microtubules they form the cytoskeleton of neurons. Three distinct polypeptides are constituents of the neuronal 10 nm-filaments, the NF-L, NF-M, and NF-H proteins. Expression of the NF-M gene appears to be correlated with projection length and arborization of neurons (Zopf *et al.*, 1987).

An *in situ* hybridization with a neurofilament M riboprobe revealed labeling in the chicken retina starting with the E6. The labeling was constantly distributed in cells of the GCL. The neurofilament M-labeled cells may be ganglion cells or displaced amacrine cells. Neurofilament antibody staining was used extensively by other groups to visualize ganglion cells and their axons in the retina of different vertebrate species (Straznicky *et al.*, 1992; Kong and Cho, 1999). By using other ganglion cell markers, it has been reported that the staining was exclusively limited to the ganglion cells. However, the expression of neurofilament may not represent a commitment to a ganglion cell phenotype (Fischer *et al.*, 2002). Bennett and DiLullo (Bennett and DiLullo, 1985a, 1985b) reported that also neuroepithelial and neuroblastic cells of the chick central nervous system express neurofilament proteins and that some of this expression is transient. The results of the *in situ* hybridization together with the literature data suggest that neurofilament M is expressed exclusively by the ganglion cells in the chicken retina, and therefore can be used as a ganglion cell marker.

Tyrosine hydroxylase

It is known that the dopaminergic neurons of the central nervous system release a large proportion of AChE. Using double fluorescent labeling it was established that in the CNS nearly all the dopaminergic neurons were labeled for AChE, and in addition there were non-dopaminergic but AChE-positive neurons (Henderson and Greenfield, 1984). In the retina, the distribution of the dopaminergic neurons was already documented. However, the

distribution of TH mRNA in the retina was not documented. Therefore, *in situ* hybridization with a TH riboprobe was done. The first TH-positive cells in the retina were identified at E12. These cells were located in the INL, next to the INL-IPL border. At E19 the TH-positive cells were placed at the border of INL with IPL, an observation consistent with previous studies. Kagami and co-workers reported that using a monoclonal or a polyclonal antibody against chicken tyrosine hydroxylase, the first labeled cells appeared at E11 (Kagami *et al.*, 1991). The phenomena of migration of TH-positive cells toward INL-IPL border was described with the help of antibody staining.

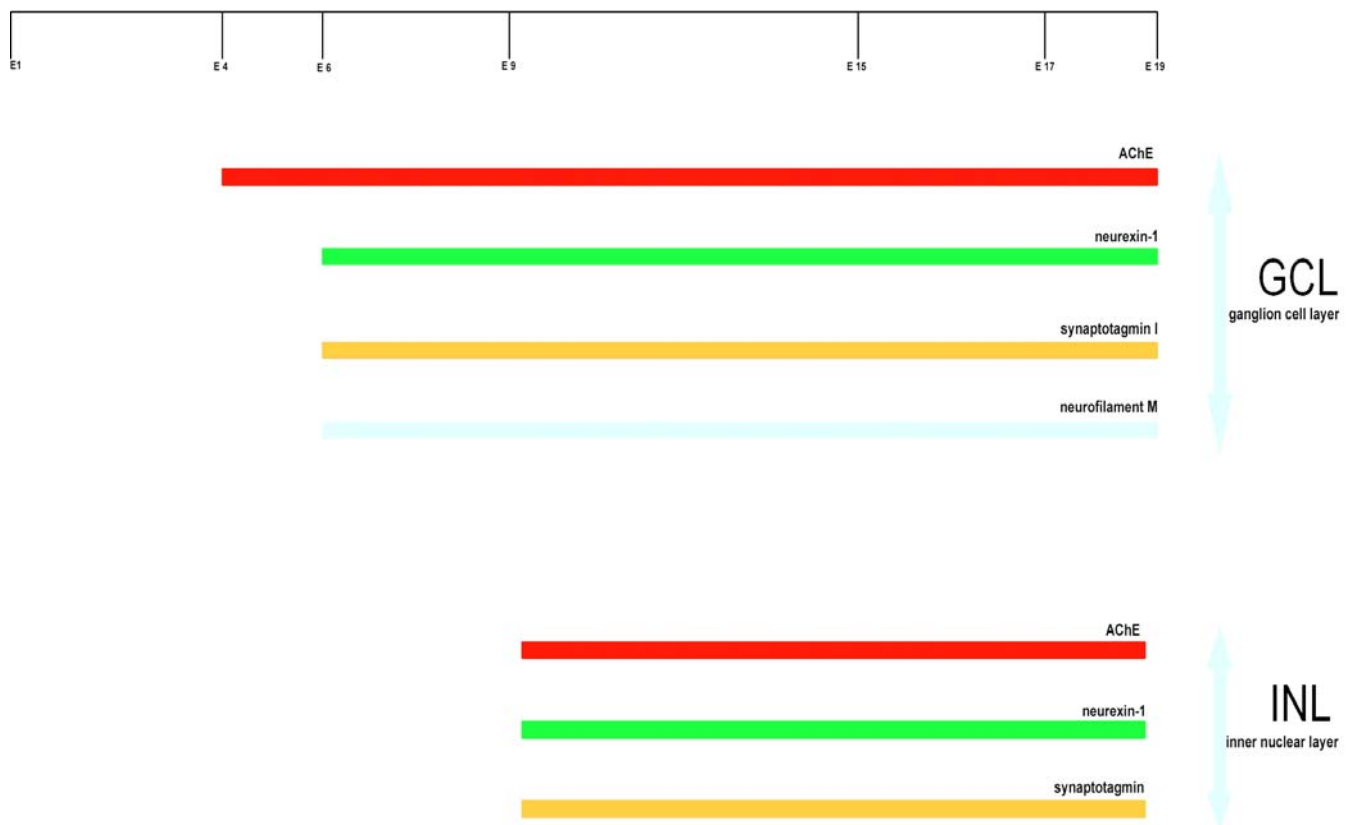


Figure 27. Scheme of the temporal and topographical distribution of AChE, neurexin-1 β , synaptotagmin I and neurofilament M gene transcripts in the developing chicken retina. Cells from the ganglion cell layer were labeled with an AChE riboprobe starting at E4, and with neurexin, synaptotagmin and neurofilament riboprobes from E6 onwards. Cells of the inner nuclear layer express AChE, neurexin, synaptotagmin mRNA from E6 onwards; they did not express neurofilament M.

4.1.3. Surface plasmon resonance and mass spectrometry-based AChE “ligand fishing”

Two approaches were used to search for unknown AChE binding partners. A first approach was the use of surface plasmon resonance technology combined with mass spectrometry to identify and characterize novel AChE binding partners. Several groups demonstrated that the BIAcore technology can be successfully used to isolate and identify target molecules from a complex biological mixture (Zhokov *et al.*, 2002). The method was applied to AChE bound to the surface of the flow cells of a sensor chip. A brain membrane extract was injected over these flow cells and the material bound to the flow cells (AChE) was recovered in a small buffer volume. However, this procedure is far from being established for the use in proteomics. The initial experiments demonstrated considerable nonspecific binding to the surfaces of the flow system. This complicated a subsequent analysis by mass spectrometry. Therefore, a genetic approach was favored over the biochemical system.

4.2. AChE-binding partners revealed in a yeast two-hybrid screen

4.2.1. Is AChE suitable for use in a yeast two-hybrid screen?

The yeast two-hybrid system was developed to identify genes encoding proteins that are physically associated with a given protein *in vivo* (Song and Fields, 1989). However, the two-hybrid system does not provide a solution for all protein - protein problems. For different experimental reasons, as discussed below, some proteins are not considered to be suited for this approach.

A major disadvantage of assaying protein-protein interactions in any heterologous system is that some interactions depend upon posttranslational modifications that do not, or inappropriately, occur in yeast. Such modifications include the formation of disulfide bridges, glycosylation and most commonly phosphorylation. This was also the case for AChE, which is an extracellular glycoprotein with three disulfide bridges. The two-hybrid system was predicted to be limited to the analysis of cytoplasmic proteins. Indeed, extracellular proteins are often N-glycosylated and contain disulfide bonds, both of which are not expected to occur in the yeast nucleus. Nevertheless, several successes were reported with transmembrane receptors. Appropriate extracellular receptor-ligand interactions were demonstrated for the growth hormone, prolactin and growth hormone releasing receptors (Young and Ozenberger 1995; Kajkowski *et al.*, 1997). These studies provided basis for the

decision to use AChE as bait for screening a cDNA library. The AChE binding partners found should however be seen in a critical way, since the protein would not be glycosylated and the interaction would be mainly depending on its primary structure.

Another obvious point of concern for the system relates to the extensive use of chimeras. The use of artificially made fusion proteins always implies a potential risk. The fusion might change the actual conformation of the bait and/or prey and consequently modify their functions. This misconformation might result in a limited activity or in the inaccessibility of binding sites. However, the use of tagged proteins in general has been very successful in many biotechnological approaches. This success might rely on the fact that protein domains can fold rather independently, enabling the co-existence of different, even artificially introduced, modules in the same protein.

Since the two-hybrid system makes use of a transcription event, one of the most crucial initial experiments was to check whether AChE was able to initiate transcription. If this is the case, it might seriously handicap the successful use of this protein in any two-hybrid approach. The LexA-AChE hybrid protein showed no activation of the reporter genes *HIS3* and *LacZ*, and therefore could be used for screening.

The two-hybrid system needs the fusion proteins to be targeted to the yeast nucleus. This might be a disadvantage for extracellular proteins or proteins that contain strong targeting signals. To avoid secretion of AChE, the 33 aa signal peptide of AChE was not included in the construct used for screening. However, rat AChE was previously successfully expressed in yeast (*Pichia pastoris*), the purified protein having properties comparable with the native AChE. The expressed AChE had a molecular weight of 67kDa (native AChE has 69kDa) suggesting a glycosylation and the same inhibition properties (Heim *et al.*, 1998). Western blots of L40 yeast cells transformed with a plasmid encoding the LexA-AChE hybrid protein showed the presence of a 75kDa protein recognized by an anti-mouse AChE antibody. Only homogenates of yeast cells collected in the logarithmic growth showed signals in Western Blot analysis with an anti-AChE antibody. These results could be explained by the nature of pVJL11 vector promoter. The normally very strong alcohol dehydrogenase 1 (ADH1) promoter in the pVJL11 vector is truncated to 410bp, and expression from this truncated promoter leads to low expression levels of fusion protein (Tornow and Santangelo, 1990).

My preliminary tests indicated that the AChE did not activate the *HIS3* and *LacZ* reporter genes and was expressed in yeast as fusion with LexA-BD. Therefore, the AChE construct was used as bait in a yeast two-hybrid screen to search for interaction partners.

4.2.2. AChE ligands in the yeast two-hybrid system

The core of AChE (present in all the molecular forms of the enzyme, aa 33-583) was used to screen a mouse brain yeast two-hybrid library. 190 colonies were found to be *HIS3* positive. From this, just 96 could activate the second reporter gene LacZ. Previous studies showed that most of the *HIS3*⁺/*LacZ*⁺ colonies were no 'in-frame' fusions and carried non-fused downstream genes encoding transcription factors (James *et al.*, 1996). Since all combinations of protein-protein interactions are assayed, the possibility of identifying artifactual partners exists, and it is a typical disadvantage of all exhaustive screening procedures. Of 96 *HIS3*⁺/*LacZ*⁺ clones, 23 were identified as 'true' AChE binding partners in the yeast two-hybrid system. Insert analysis was performed first. From 96 clones just 60 had a library insert. The remaining 60 clones were retransformed with positive and negative controls to assay the specificity of the interaction. These included cotransformation of the detected prey with the empty vector (containing LexA-BD) or with a vector encoding an irrelevant protein (p53 and lamin). Further false positives were identified, e.g. library cDNAs that activated the reporter genes by itself, or 'sticky' fragments that could interact with the control proteins p53 and lamin. One of the yeast two-hybrid pitfalls is the high number of false positives. However, it was possible to partly eliminate them by a series of additional transformations and testing the reporter gene activity.

More information was gained by sequencing the prey cDNA. Analysis of sequences revealed other possible false positives. This category included all known genes that are not in frame with the activation domain and those with unknown genes that show a polyAAA tail indicating that the gene is probably inverted. Other genes that might be omitted from further analysis do not encode proteins but RNA. Some proteins can also be excluded if they are part of a list of known false positives that are frequently found, independently of the target used. This list includes hsp's, ribosomal proteins, cytochrome oxidase, mitochondrial proteins, proteasome subunits, ferritin, tRNA synthase, collagen-related proteins, Zn-finger proteins, vimentin (from the web site of E. Golemis <http://chaos.fccc.edu/research/labs/golemis>).

The AChE interaction partners identified in the yeast two-hybrid screen were classified in two main categories and are listed below:

1. **Unknown cDNAs:** unknown mouse cDNA, no homologies; mouse DNA from clone RP 23 143 (3X); mouse DNA clone RP 23 - 56 on chromosome 18 (2X); mouse DNA from clone RP 23-415c3; mouse DNA from clone RP 23-354 K2; mouse DNA from clone RP 23/actin gamma cytoplasmic; mouse embryo 12 days DNA.

2. **Known proteins:** mouse septin; mouse laminin beta1; Siat7f protein, sialyltransferase; rabphilin 3A; N-acetyllactosaminide alpha-1,3-galactosyltransferase; protein kinase beta subunit; mouse Tbk1 (2X); adult male cerebellum cDNA - hypothetical Zn-finger CChC type containing protein; mouse creatine kinase (2X); mouse, similar to HBx Ag transactivated protein 2.

Figure 28 presents an overview of the screen results and the ligand localization. The basis for protein sequence alignments was provided by the open reading frame of the vector. One of the advantages for databases research was the work with mouse proteins, as extensive information was available about the mouse genome. Only the homologous proteins from mouse were considered and further analyzed. Nevertheless, the results of a database research should be carefully interpreted, also because the clones did not include full-length DNAs.

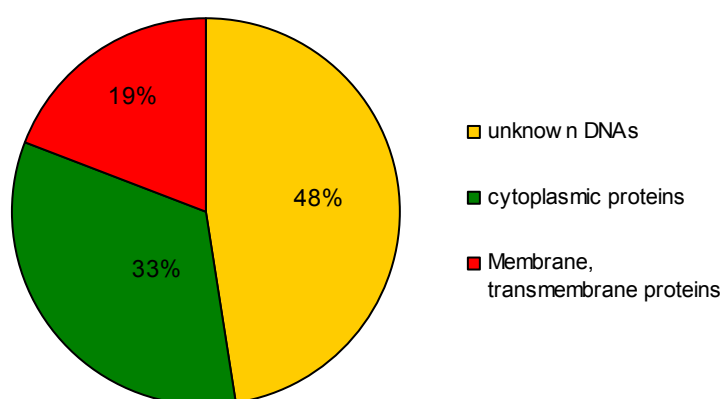


Figure 28. Schematic representation of the yeast two-hybrid screen results.

Although the choice of which interactions to characterize further is arbitrary, some guidelines were used. For *new proteins* that show no homology with any known sequence in DNA or protein databases, the above selection becomes less trivial. As general criterion, the redundancy at which the interaction is found is considered to be a clue for the importance of the interaction. For *known proteins*, the potential biological relevance of the interaction is the most important criteria.

Table 3 summarizes the known proteins found as AChE ligands in the yeast two-hybrid screen, together with their localization, functions, and possible relevance of the interaction.

Name of AChE ligand	Subcellular localization	Function	Biological relevance of the interaction
Adult male cerebellum cDNA; hypothetical Zn-finger CChC type containing protein	<ul style="list-style-type: none"> nucleus 	Nucleic acid-binding protein	Often present in the list of false positives
Creatine kinase	<ul style="list-style-type: none"> cytoplasm mitochondria 	Energy and metabolism, phosphotransferase	?
Protein similar to HBx (hepatitis B virus protein X) Ag transactivated protein 2	<ul style="list-style-type: none"> nucleus cytoplasm 	Transcriptional transactivator, activation of signal transduction pathways	List of false positive
Laminin-1 beta	<ul style="list-style-type: none"> extracellular matrix 	Cell growth, differentiation, neuron migration, growth, morphology, and adhesion,	Relevant
N-acetyllactosaminide alpha-1,3-galactosyltransferase	<ul style="list-style-type: none"> membrane of Golgi trans cisternae 	Enzyme EC 2.4.1.87	Glycosylation of AChE?
protein kinase beta subunit	<ul style="list-style-type: none"> cytoplasm 	Signaling pathways	Other researchers could show an AChE-PK co-regulation. Direct binding?
Rabphilin 3A	<ul style="list-style-type: none"> cytoplasm attached to synaptic vesicles 	Binds to rab3a which is a GTPase that mediates directional vesicular trafficking and regulate synaptic vesicle exocytosis	?
Septin	<ul style="list-style-type: none"> presynaptic vesicles membranes, 	GTPase enzyme, required for the cytokinesis stage of cell division and for exocytosis	?
Sialyltransferase	<ul style="list-style-type: none"> membrane of Golgi apparatus, endoplasmic reticulum 	Cellular communication and signal transduction	Glycosylation of AChE?
Tbk1	<ul style="list-style-type: none"> cytoplasm, translocated to nucleus 	Triggers the host antiviral response to viral infection	?

Table 3. Yeast two-hybrid AChE ligands: localization, function, relevance of the interaction.

Another factor that has to be considered after the analysis of sequences is the possibility of the two proteins to find each other in the natural environment. Table 3 presents the localization of these ligands in the cell. Due to the so-called time/space constraints it is potentially possible that both proteins, although able to interact, are never in close proximity to each other within the cell. The two proteins could be expressed in different cell types, or even when found in the same cell they could be localized in distinct subcellular compartments. Moreover, interacting proteins can be expressed at different points during embryogenesis or during homeostasis (e.g. at different time points in the cell cycle). So once two interacting partners are identified, the biological relevance of this interaction remains to be determined.

4.2.2.1. Unknown cDNAs - new proteins

Interestingly, 48% of the prey obtained by screening a yeast two-hybrid library with AChE was represented by unknown cDNA sequences. The analysis of these sequences is more difficult compared with the known proteins and raised interpretation problems due to the lack of an adequate amount of information. Nevertheless, the unknown clones will certainly give rewarding outcomes. As already mentioned, the frequency at which the interaction was found might be a clue for the importance of the interaction. This was the criteria that allowed a primary selection. Important to mention is that this frequency is not related to the strength of the interaction. With new proteins, it was difficult to find parts of the coding sequence. By performing extensive database searches, and using multiple sequence alignment algorithms, one can sometimes reconstitute full-length cDNA clones (using FASTA and *blastn*). Using these sequence data, one can perform many different algorithms to predict subcellular localization, transmembrane domains, conserved functional patterns, etc. This could be done with PROSITE (USA) and PROPSEARCH at EMBL. However, the attempts to reconstruct full length cDNA or to identify motifs were not very successful. Of course, such predictions are never 'direct' evidence, but could be the starting point for more conclusive experiments.

In this study, the analysis of the potential AChE interaction partners was limited to known proteins mostly because these proteins were less problematic to investigate. However, the novel proteins that interact with AChE can prove to be more fascinating and bring new insights into the adhesion function of AChE.

4.2.2.2. Cytoplasmic proteins

AChE is an extracellular soluble or membrane bound protein. A majority of 33% from the known proteins identified as binding partners for AChE were localized intracellularly (see Table 3). Taken together, these facts would suggest that the cytoplasmic proteins should be considered as 'false positives'. Indeed, a classification of potential protein – protein pairs based on known function or location is normally used as a criterion to decrease the number of false positives in a yeast two-hybrid system. It is usual to avoid further studies of combinations that are unlikely to colocalize in vivo. There are exceptions, however, in which some proteins may move to different parts of the cell, where they may interact with other proteins.

Surprisingly, it was observed that under stress conditions, mice produced the rare read-through AChE splice variant and this variant accumulated in neuronal cell bodies (Birikh *et al.*, 2003). This came unexpected, considering the existence of a signal peptide on the nascent AChE and the generally secretory nature of this protein. Moreover, Ye and colleagues (Ye *et al.*, 2001) could show that nascent polypeptides targeted for ubiquitin-dependent processing and destined for secretion may be released from the rough endoplasmic reticulum into the cytoplasm. Soreq and co-workers suggested that under stress the intracellular compartmentalization could be altered, and thus AChE could also appear intracellularly. However, there is no experimental evidence available to support this and it should be still considered as a speculation.

Secondly, like other glycosylated membrane and secreted proteins, AChE is synthesized at the level of rough endoplasmic reticulum and translocated into the lumen where their signal peptides are cleaved. The enzyme is first synthesized as inactive precursor, acquiring activity in the reticulum (Hassig *et al.*, 1991). Only a fraction of the enzyme is transported towards the cell surface, a large proportion of AChE subunits, either active or inactive, are degraded in non lysosomal compartments (Massoulie *et al.*, 1993). One can consider that part of this inactive AChE precursor is released into the cytoplasm and accounts for the interaction with cytoplasmic proteins.

The intracellular AChE binding proteins could be classified as follows:

1. Transcription factors - A hypothetical Zn-finger CChC-type containing protein and a protein similar to HBx (hepatitis B virus protein X) Ag transactivated protein 2

These two proteins are involved in processes related to transcription events, and are localized in the nucleus. One of the most important and better understood types of

transcription factors are zinc finger proteins. The Zn-finger motif is often present in proteins that bind nucleic acids. HBx is also transcription transactivator. Transcription factors appear often in the lists of yeast two-hybrid screen false positives, due to their ability to bind and activate gene promoter elements. Therefore, these two proteins were considered to belong to the list of false positives.

2. Synaptic proteins - Rabphilin 3A and septin

Some of the cytoplasmic proteins identified in the yeast two-hybrid screen, e.g. rabphilin 3A and septin, are proteins involved in synaptic functions like vesicle trafficking and exocytosis (Hsu *et al.*, 1998; Fung and Scheller, 1999; Hsueh *et al.*, 2000).

Rabphilin 3A was the first identified rab3a effector (Shirataki *et al.*, 1993) binding to rab3a through its N-terminus (Schlüter *et al.*, 1999). Rab3a is a member of a large family of low molecular weight GTPases that is localized to synaptic vesicles, and is thought to mediate directional vesicular trafficking and regulate synaptic vesicle exocytosis. Rab3a cycles between an active, GTP-bound form and an inactive, GDP-bound form, which is dependent upon the accumulation of rabphilin 3A which stabilizes the GTP-bound form of rab3a on the vesicle (Hsueh *et al.*, 2000). Rabphilin 3A binds also to CASK, a scaffolding protein. CASK binds to Mint-1 and Veli-1 forming a presynaptic multicomplex involved in the recruitment of channels and receptors to the neurexin-1 β & neuroligin-1 complex at the forming synapses (Zhang *et al.*, 2001).

The septins are a family of GTPase enzymes, some of which are required for the cytokinesis stage of cell division and others for exocytosis. Nine mammalian septins have been found, however only G-septin is found to be phosphorylated. Nedd5 is a brain specific septin and its phosphorylation increases in response to stimulation with cyclic nucleotides in nerve terminals. Nedd5 immunoprecipitated with the SNARE protein syntaxin by directly binding to syntaxin via the SNARE interaction domain. These data suggest that septins may regulate vesicle dynamics through interactions with syntaxin (Beites *et al.*, 1999). In mice and humans, 12 septin genes generate dozens of polypeptides that form hetero-oligomeric filamentous complexes. These filaments, which organize higher-order structures by self-assembly and templated assembly, are regarded as the functional units of the septin cytoskeletal system (Kinoshita *et al.*, 2002).

How does AChE get in contact with rabphilin 3A or septin? As mentioned, AChE can appear intraneuronally under stress conditions. During periods of cell differentiation or synaptogenesis, many of the synaptic components are synthesized long before they become functional. It is confirmed that these components can actively contribute to

synaptogenetic steps and are recruited from cytoplasmic pools (Garner *et al.*, 2002). AChE is present very early in developing neurons and secreted into the extracellular space. However, during these early developmental steps, the synaptic secretory machinery is far from being well organized. One can consider that during these intense activity steps not all the proteins, e.g. AChE, reached their classical subcellular localization or functions. With AChE present in the neuronal cytoplasm, an interaction between AChE and rabphilin3a or septin will raise interesting possibilities that AChE might be involved in synaptic vesicle dynamics. Electron microscopy studies of subcellular localization of AChE in the post-mitotic neurons are required in order to establish whether this model can be confirmed. Co-immunoprecipitation experiments would also enable to determine if the binding appears outside the yeast system. Another possibility is that the existence of some structural motifs on the proteins may be responsible for this interaction. However, there is the possibility that the interaction of AChE with rabphilin 3A and septin in a yeast two-hybrid system could be the result of a forced colocalization of the proteins in the same cellular compartment. Nevertheless, AChE is not the sole extracellular protein found to interact with septin. Other extracellular proteins, e.g. sip1, were identified as binding partners for septin in yeast two-hybrid screens (Giot *et al.*, 2003).

Figure 29 summarizes the model in which cytoplasmic AChE, via binding to rabphilin3A or septin may modulate synaptic vesicle dynamics on developing neurons.

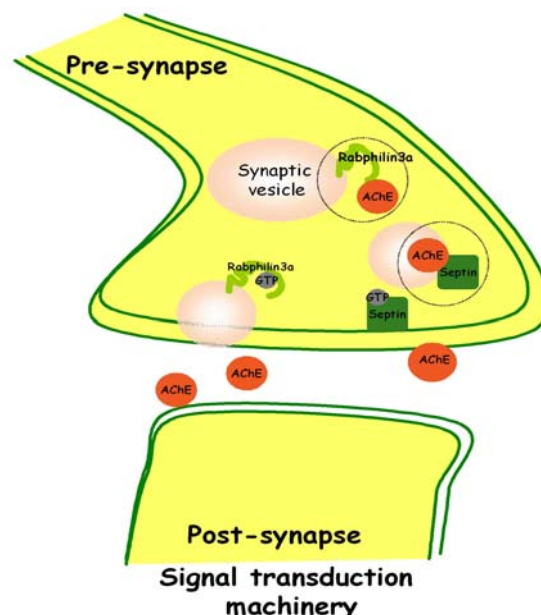


Figure 29. Schematic drawing of the synaptic AChE binding partners. Rabphilin 3A and septin are pre-synaptic proteins connected to synaptic vesicles and involved in vesicle trafficking. AChE is membrane bound or secreted, but can be also localized cytoplasmatically, and therefore may interact with rabphilin3a and septin.

3. Kinases – *Tbk1* kinase, protein kinase- β and Creatine kinase

There are accumulating data that show a connection between cholinesterases and kinase pathways. Therefore, the yeast two-hybrid results regarding kinases are very promising. AChE is shown to interact with the scaffolding protein RACK and through it, with its target, protein kinase C- β II (Birikh *et al.*, 2003). Moreover, an overexpression of AChE through transfection studies lead to an increase in the expression of protein kinase C (Keller *et al.*, 2001). As already mentioned, AChE is related to processes of neuronal differentiation, neurite outgrowth and synaptogenesis. Kinases are well established players involved in processes like proliferation, differentiation, and apoptotic cell death.

Three different kinases were found to interact with AChE in yeast: TANK-binding kinase, protein kinase A and creatine kinase. TANK-binding kinase is a protein that mediates the TANK's ability to activate NF- κ B (Pomerantz and Baltimore, 1999). NF- κ B is a ubiquitously expressed transcription factor that regulates the induction of genes involved in immune and inflammatory cell function (Verma *et al.*, 1995). Not too much is known about the roles of the TANK-kinase during development of the nervous system.

Creatine kinase is an enzyme involved in energy metabolism in brain and muscle. It was revealed that migrating neuronal growth cones exert traction forces that are generated by ATP-driven F-actin/myosin interactions. Sustained generation of these forces requires an energy supply mediated by the guanidino kinases, creatine kinase and arginine kinase (Wang *et al.*, 1998). Creatine kinase is a key ATP-generating enzyme that regulates ATP within subcellular compartments. It could be possible that creatine kinase is bound to AChE where it may provide bursts of site-specific high-energy phosphate necessary for developmental processes, e.g. efficient receptor signal transduction during cytoskeletal reorganization.

The interaction of AChE with a third kinase, the beta subunit of a cAMP-dependent protein kinase A seems more pertinent at the neuromuscular junction. This due to the accumulating data that show a connection between cAMP and AChE levels at the junction. Tsim and co-workers showed that in the developing neuromuscular junctions, the regulation of muscle AChE expression can occur by two mechanisms: factors secreted from the presynaptic neuron during synaptogenesis and muscle intrinsic factors during myogenesis (Siow *et al.*, 2002). Their results suggested that muscle intracellular cAMP is one of the candidates to regulate the expression of AChE during early myogenic differentiation. The activation of intracellular cAMP synthesis is mediated by a cAMP-dependent protein kinase (PKA) and a cAMP-responsive element-binding protein (CREB) (Choi *et al.*, 2000). The decrease of cAMP may be a signal to trigger the differentiation process such as the

induction of muscle-specific genes that includes the expression of AChE. By binding to AChE, the cAMP-dependent protein kinase would be a sensor for AChE concentrations and regulate its own expression.

4.2.2.3. Membrane proteins

N-acetyllactosaminide alpha-1,3-galactosyltransferase and sialyltransferase

The two enzymes are transmembrane proteins found in the trans-cisternae of Golgi apparatus. These proteins present enzymatic functions and are involved in processes of N-glycosylation. AChE is a glycosylated protein, though the functions of the glycoparts are largely unknown. An affinity of AChE to these enzymes is therefore to imagine. Galactosyltransferase catalyzes the transfer of galactose from UDP-galactose to an acceptor molecule using manganese as cofactor. Sialyltransferase catalyzes the polycondensation of alpha-2,8-linked sialic acid required for the synthesis of polysialic acid, a modulator of the adhesive properties of neural cell adhesion molecule (N-CAM1). The encoded protein, which is a member of glycosyltransferase family 29, is a type II membrane protein that may be present in the Golgi apparatus.

In conclusion, the yeast two-hybrid screen revealed that AChE binds to enzymes that are involved in glycosylation, one of the AChE maturation processes. We can interpret this as a transient interaction of the inactive AChE polypeptide that appears in Golgi apparatus.

4.3. Laminin-1 interacts with AChE

Using a yeast two-hybrid screen, we were able to identify an 898 bp fragment of laminin-beta1 chain as interaction partner of AChE in yeast. Co-immunoprecipitation was used to confirm the interaction between laminin-1 and AChE. The β -galactosidase filter lift assay data indicated that the interaction between these two proteins is moderately strong and that the region containing 240th aa residue to 503th aa residues of AChE is essential for interaction (the numbers include the 32 aa of the signal peptide). However, when transformed in yeast together with laminin-1 beta, the AChE construct lacking the catalytic serine 234 showed a weaker activation of the LacZ reporter gene than the constructs that included Ser 234. That the catalytic serine is not important in the interaction came not surprisingly. It was already demonstrated, using specific catalytic site inhibitors, that the developmental functions of AChE e.g. neurite outgrowth and differentiation are independent of its enzymatic activity. Johnson and Moore previously reported that human AChE binds to

mouse laminin-1 (Johnson and Moore, 2003). A significant reduction in the binding was observed when peripheral anionic site inhibitors (fasciculin, propidium, BW284c51) were used. Fasciculin, a 61 aa peptide, reduced AChE binding to laminin-1 close to zero. Therefore, it was proposed that the binding occurs at the peripheral anionic site of the enzyme (Johnson and Moore, 2003). The yeast two-hybrid results indicate that by partially disrupting the structure of the peripheral anionic site, the interaction appears to become weaker. However, studies with AChE mutants of the amino acids that comprise the peripheral anionic site are necessary for determining if the peripheral anionic site is directly involved and which amino acids are essential for interaction.

The coimmunoprecipitation studies indicated that the interaction was also highly dependent on the NaCl concentration used in the interaction buffer. High ionic buffers (250 mM to 500 mM NaCl) did not allow binding of these two proteins. These results confirm the observations of Johnson, 2003, indicating that an electrostatic mechanism may be involved in binding. Electrostatic complementarities between interacting proteins has been found to be one of the major driving forces for complex formation (McCoy *et al.*, 1997). Examination of the 3-D structure of AChE showed the enzyme to be characterized by a marked asymmetric spatial distribution of charged residues, which were shown to roughly segregate into a 'northern' negative hemisphere and a 'southern' positive one (Ripoll *et al.*, 1993). The asymmetric distribution of surface potentials was thought to be essential for the fast catalysis of AChE. However, it has been shown that the role of surface potential in catalysis is only minor (Felder *et al.*, 1997). It was proposed that the surface potentials are related to other functions than catalysis, e.g. adhesion (Botti *et al.*, 1998). Analysis of the sequence alignment of AChE, neuroligin, neurotactin, gliotactin showed no sequence motif shared exclusively by these proteins. Thus, it was concluded that the above mentioned proteins are the members of a class of adhesion molecules that, because of their common electrostatic motif, are named electrotactins (Botti *et al.*, 1998). Therefore, the high interaction dependence of the salt concentration supports a contribution of an electrostatic mechanism to the interaction.

Which is the site of interaction on laminin-1? It is known that laminins are fibrillar glycoproteins. Figure 30 shows that laminin-1 is a heterotrimeric protein composed by α 1-, β 1-, γ 1- chains, with the three chains forming a cruciform structure. The short arms of the cross-like structure are represented by the N-terminal regions of the three chains, while the long arm is a coiled structure of the C-terminal regions that holds them together.

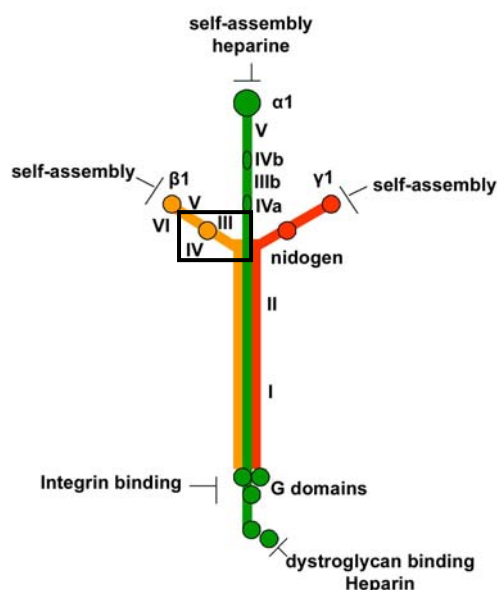


Figure 30. Domain structure and interaction sites of laminin-1. The laminin fragment possibly interacting with AChE is marked.

A single fragment comprising 898 bp of laminin-beta1 chain was found to interact with AChE. The fragment is located closer to the N-terminal region of the chain and includes the globular domain IV (see Figure 30). The N-terminal regions of the laminin chains are heterogeneous, consisting of alternating globular and cysteine-rich domains of varying size and number (Tunggal *et al.*, 2000). The $\beta 1$ chain includes two globular domains named IV and VI (Figure 30). The N-terminal globular domain VI of the $\beta 1$ chain is important in laminin polymerisation (self-assembly) (Yurchenco *et al.*, 1992) and the globular domain IV has no specific function. Utani *et al.*, (1997) reported that fibulin-2, an extracellular matrix glycoprotein, binds to the globular domain IV on the chain $\gamma 2$. The globular domains alternate with non-globular domains V and III. These domains are formed of 3-8 LE modules, rich in cysteine (8 residues) and homologous to epidermal growth factor. The 300 aa of the laminin-beta1 fragment identified by yeast two-hybrid screening comprises the globular domain IV and part of the domain III. With respect to which site on laminin-1 β might interact with AChE, it is likely that the globular domain mediates this binding.

The binding of AChE to laminin-1 clarifies the AChE postulated role of an adhesive molecule during development. It is reported that laminin-1 binds to different integrin receptors like $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 7\beta 1$, $\alpha 6\beta 4$ (Teller and Beaulieu, 2001). Integrins comprise a large family of cell adhesion molecules that mediate interactions between the extracellular environment and the cytoplasm. The cytoplasmic domains of integrins interact with microfilaments. Therefore, integrins link the cytoskeleton to the extracellular matrix. The site of this interaction between microfilaments and the extracellular matrix is called a focal

contact. These focal contacts provide the strength necessary for the cell to arrange the extracellular matrix by pulling on it. In addition, the focal adhesion sites are the location of an activated enzyme called focal adhesion kinase. FAK is a tyrosine kinase that appears to be activated by certain integrins. Thus, integrins are a very important way of promoting signal transduction in the cell. Integrins can stimulate other signaling receptors as well.

Despite the large amount of indirect experimental evidence supporting the involvement of AChE in adhesion processes, no evidence was provided to support this. Here we propose a model for the developmental function of AChE, based on its interaction with laminin-1. The most likely mechanism through which AChE sends signals into the cell is the following: during development, the early secreted AChE binds to laminin-1 β (see Figure 31). Laminin-1 β is expressed very early and ubiquitously during development. The laminin-1 β mRNA was first detected at two or four cells stage (Dziadek and Timpl, 1985) and the mouse laminin-1 protein first at the morula stage (Cooper und MacQueen, 1983). Laminin-1 binds to integrin receptors, a class of cell adhesion molecules known to be involved in neuronal migration during CNS development, most likely by mediating the adhesive interactions between neurons and radial glial fibers. It is well established that cell adhesion molecules such as integrins play an important role in building and maintaining synaptic structure during CNS development (Benson *et al.*, 2000). Integrins activate the ERK activation cascade (Ras \rightarrow Raf \rightarrow MEK \rightarrow ERK) via a focal adhesion kinase mechanism (see Figure 31). In the CNS the kinase involved is named pp125FAK.

Such a mechanism was proposed for transglutaminase, a cell surface glycoprotein with enzymatic activities that also promotes adhesion independent of its enzymatic activity (Akimov *et al.*, 2000). Transglutaminase binds both fibronectin and β 1-integrins promoting integrin clustering and intracellular signal activation.

Thus, the intracellular effect of AChE would consist of exerting changes in signaling pathways, and via remodeling of cytoskeletal structures.

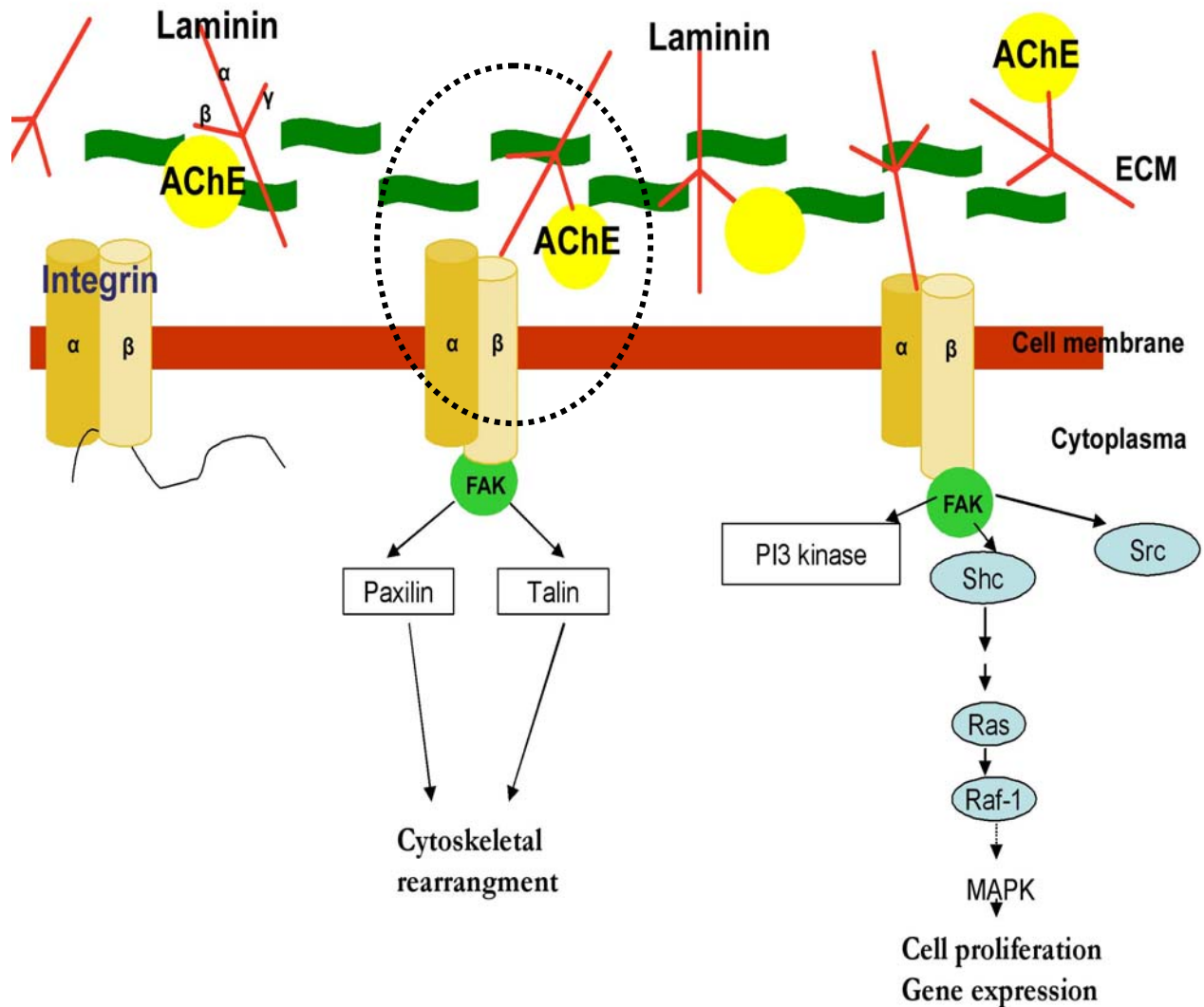


Figure 31. Proposed model for AChE signaling. AChE binds to the laminin-β1 chain and through laminin-1 can enhance the activation of integrin receptors. Integrins, as adhesion receptors, are able to send signals into the cytoplasm and via FAK activate intracellular signaling pathways. ECM-Extracellular matrix, FAK-focal adhesion kinase, MAPK-mitogen activated protein kinase, PI3-phosphatidylinositol-3 kinase.

4.4. Future work

The studies on binding partners of AChE present many opportunities for future experiments. The challenge for future studies is to demonstrate the biological significance of these interactions.

A primary goal of future research should be to determine whether the proteins found as AChE binding partners in a yeast two-hybrid screen interact with AChE also in a biochemical system. Once the interaction is confirmed in a biochemical way, the kinetic parameters of the binding (KD) and the part of the proteins involved in interaction would be determined.

For the unknown proteins a first step would be to clone the entire sequence of the gene by screening a mouse cDNA library and RACE-PCR, then computer-base analyze the characteristics of the encoded protein, and study its expression pattern with *in situ* hybridization. After determining the molecular properties of the protein, their binding to AChE would be analyzed. Tagged constructs (His, IgG, etc) or antibodies against the new proteins would be generated for coimmunoprecipitation experiments.

A further advance would be to determine the significance of the interaction in the cell. This will require disrupting the interaction of the two proteins (siRNA, antisense transfection for AChE) and assessing the subsequent effects on AChE functions, such as adhesion. A future task will be to unravel in detail the molecular cascades involved. Detailed studies of the Ca^{++} - or cAMP-mediated signaling pathways components under modified AChE expression will be helpful in further elucidating the cholinesterase signaling.

Although these studies represent a challenge, they are likely to result in rewarding advances in the field of AChE research.

5. Summary

Acetylcholinesterase (AChE) is the enzyme that hydrolyses the neurotransmitter acetylcholine at the cholinergic synapses. Besides this principal role, called the classical function, AChE shows also other non-classical functions related to processes during embryonic development and diseases. The existence of multiple molecular forms, the homology with other neuronal cell adhesion molecules, and the early expression pattern, suggest that AChE may function in cell adhesion, and thus in neurite growth, guidance and even synaptogenesis.

To gain an insight into the noncholinergic, cell adhesion promoting functions of acetylcholinesterase, we sought proteins interacting with it. Since AChE is mostly an extracellular or membrane- attached molecule, the binding partners for AChE should be: expressed in the same tissues and at the same developmental stages as AChE, have both an extracellular and an intracellular domain connected through one or more transmembrane domains, and possibly be able to initiate an intracellular signaling cascade.

Due to the 34% sequence identity between AChE and neuroligin-1, a neuronal cell adhesion molecule, we postulated that the neuroligin's binding partner, neurexin, may bind to AChE. Initially, we had to show that the AChE binding partner is expressed in the same tissues and at the same time with AChE. The pattern of expression of neurexin-1 β and AChE was analyzed in the chicken retina using *in situ* hybridization. The results show that AChE-positive cells in the retina also coexpress neurexin, with AChE preceding neurexin (~48h). Both are strongly expressed long before synaptogenesis, which starts about embryonic day 13 in the chicken retina. Their expression profiles are consistent with the hypothesis that the neurexins and AChE have a function in early neuronal differentiation and axonogenesis. However, no specific neurexin-1 β binding to AChE was evident using surface plasmon resonance (Comoletti *et al.*, 2003).

In the second part of the work, a yeast two-hybrid screen was initiated aimed at identifying novel proteins that interact directly with AChE. 2×10^6 clones from a cDNA mouse brain library were screened with a bait containing exons 2, 3 and 4 from ACHE mouse gene. 190 His positive clones were identified and from these clones 96 were also positive on β -galactosidase test. The plasmids were isolated, re-transformed in *E.coli*, and controlled for the presence and size of the cDNA insert. From 96 clones, we thereby identified 23 prey clones that 1) grew well on histidine-free plates and displayed strong β -galactosidase activity, when coexpressed with the bait, and 2) showed no interaction with the reporter genes in coexpression with LexA binding domain or with control fusions lamin-LexA binding domain and p53-LexA binding domain. This strongly suggested that the proteins encoded by these clones are binding partners of acetylcholinesterase in yeast.

Sequencing of the cDNA inserts of the clones identified 18 independent candidates: 7 are unknown proteins and 11 are already known mouse proteins. From the 11 known mouse proteins, 4 were membrane and extracellular proteins, 2 were transcription activators, 2 were synaptic proteins and 3 were kinases. Due to its subcellular localization, structure and functions, we first focused on showing that laminin is binding to AChE in another system than yeast.

Confirming the interaction between AChE and its binding partners outside yeast is the first step after a screen. By a yeast two-hybrid approach, we identified an interaction between the AChE and an 898 bp fragment of laminin-beta1 chain, an extracellular matrix protein involved in neuronal differentiation and adhesion processes. The yeast two-hybrid data indicated that the interaction between these two proteins is moderately strong and that the region containing 240th amino acid residue to 503th amino acid residues of AChE is essential for interaction. The interaction was confirmed by co-immunoprecipitation assays. In addition, the co-immunoprecipitation showed that the binding was highly dependent on the NaCl concentration used in the interaction buffer. These results confirm the observations of Johnson (Johnson and Moore, 2003), indicating that an electrostatic mechanism may be involved in binding. This led to the model in which AChE is binding to laminin-1 during early developmental stages. Laminin-1 interacts with integrin receptors (Teller and Beaulieu, 2001) and is so able to send signals into the cell modulating signaling cascades.

Taken together, the results of this present study suggest a role for AChE in heterophilic adhesion. Understanding this role will bring a major progress in the field of non-classical functions of AChE.

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APPENDIX

Transmembrane 4 superfamily, member 2 (tetraspanin)

CCCNCCAAACCCAAAAAAGAGATCTCTATGGCTTACCCATNCCATGTTCCAGATTACGCTAGCT
TGGGTGGTCATATGGCCATGGAGGNCCCGGGGNATCCGAATTCGCGGCCGCGTCGACATCGAGGAG
AATGGAGACCAAACCTGTGATAACCTGTCTCAAAACCCCTCCTCATCATCTACTCCTTTCGTCTTCTG
GATCACTGGGGTGATCCTGTTGGCCGTGGAGTCTGGGGAAAGCTGACTTTGGGAACCTATATCTC
CCTGATTGCTGAGAACTCCACAAATGCTCCCTATGTGCTCATTGGAACCGGCACCACCATCGTGTT
TTTTNGTCTCTTTGGATGCTTTGCTACATGCCGTGGTAGTCCATGGATGCTGAAACTGTATGCCAT
GTTCTGTCCCTGGTGTTCCTGGCTGAGCTTGTGCTGGCATTCTGGATTTGTGTTTCGTTCATGA
GATCAAGGACACCTTCTGAGGACTTACACGNATGCCATGCAGAACTACAATGGCAATGATGAGA
GGAGCCGGGCTGTGGACCATGTGCAGCGCAGCCTGAGCTGCTGTGGCGTGAGAACTACACCAACT
GGGAGCAGCAGCCCCTACTTCTGGAACCATGGCATCCCCCCCCAGTTGCTGCATGAACGAAACTG
ACTGCAATCCCCCTGGATCTGCACAATCTGACTGTGGCCGNCCACCAAAGTTAATCAGAANGGCTG
GTTATGATCTGGGTGAACAAGGTTTTTTGGAAACTAACATGGGGNATCATTTGCTGGGNGNGGNA
TTTGGNANTNGCGTTCTA

Rabphilin 3A

TNNGNTGNTGNAGATACCCACCAAACCCAAAAAAGAGATCTCTATGGCTTACCCATCCCNTGT
NCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGNCCCGGGGATCCNAATTCGCGGCC
GCGTCGACCGGGTCCGGTGCAACGAGCAGAGGCGCCGCCCGCGGGAATGTGAACGAAGAGCCAC
CGGCCGCGCCGCAACCGGGTCCGTGCCGATTTGATGGGACGGGCCCGCGGGGGAGGCTCGTGAGG
CCGCCGCTGCCACTGCCGGAGCGCTGAGGTTAGGGGCCAGCCGAGAGGCCTAGAAACACTGCCGC
TACCGCGGAACCGGAGGGACGTCNNTTCGGACGGCCGTAGTAGCCCGGGGCTCTCCGCCGCTGCC
TGGACCTCCCCCGTGCGTCCCGGGCCGTGCCCCGCCCCCGCGCGNCCCTAGCTCGCAGCCCA
CTCCGCGCAGCCACAGATCATCTTCCGATTCTTTCCAGGAGCTTCAAGTAGGGATATGTCCTCA
GCACCAACTACTCCTCCATCAGTGGGATAAAGTAGACGGGATTTTCTCGGAAGTCCGTCAGAAAA
AGCCAGACAGAAGAGGTGCAAAAGCTCCTCCCAGTTTCAGGTCTCAAGGCAAGCCANTTGAGCT
CACGCCTCTGCCACTGCTGGAAAGACGTTNCNAACNNTCANAAGCAGCCTTGAAACTTGTTTCCT
NAAAGAACTTCANCAGGGGCTGGTGTCCATTTTTTTGGACCTTCATGGGANCNCGCCTATTCTGA
TTNTTAAAAATGGAAAAGAATACAAGGCGGGCTTCNCTNTCTTTTAATTGAAACTNGGGTGGGG
AANTTACNATTTNACAAATTA

Septin (Nedd 5)

TACGATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGCCCGGGGATCCGAAT
TCGCGGCCGCGTCGACCCTGACGAGCGTTCTCCCGCGTGTGAGCGGATTAGACAGGNAAAATCA
AAGAAAAGATTGTTTTAAGGCNACCAACCAACTTTNTTTTTTTTAAANNNCNAAAAACCCCTGGG
CTNTTGTNNGNATTTTNCNAAANTTTNCCCCCAATNCAANN'TN

*N-acetyllactosaminide alpha-1,3-galactosyltransferase
Galactosyltransferase*

TCTATTTCGATGATGAAGATACCCACCAANCCCAAAAAAAGAGATCTCTATGGCTTACCCATACG
ATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGCCCGGGGATCCGAATTCG
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TGGGGCTGAGTAGTAAGATCATGTGAATGCCTTGAGAGCCCCACTCTCCTCAGCAGAAGCGCACG
GCTGGAGAACTGGGAGTCTCCCCACACGGTGAAGGGTGGAAACCAAAGTTTCACCATCTCTCTGC
CACTGCACAACACAGTGCAGAGCTTGACACTGTGCTGCAGTTTCANATCCAGGTCTCCACATGGA
GGCTTTGGGAACGAGGGGTGAGAGTGGATGGGCAGTGCCGACTTTGACAGCCTGAAGAGGATCTC
AGTCAGATGAGAAACAGCTTGATCCAGGGCCTGAAAGATTAGCTGTAAGTGGCCACGTCC

CTAAAGACCGTCACTTTCTAGCTTGCTTCTGAGAAGGGGTGGACCAGCTGACTTTCCATGGAGAG
GGTTGGTGACAATGCCTTTGGTGGTTCCCTCGTGCTNGTAGATCTGAATCCACTCTGCCCCCN
TGCAACCTTTTTCATTTGCTTTTTTGTGTTTGGTTGGTTTGGCTTTTGCTTCTGGTTTTTGTGTTGGT
TTTTTGTGTTTCTGGCCGNACTCTTTGGCAAAGGCAAGAGCANNTGCTGTTACAGGGCAGGCCAG
CCCCACCTGGGNCTCTTGGCTACTGGATAGCCTCTTNCNATCN

Syalyltransferase

AATACTATCTTTTCGATGATGAAGATACCCACCAAACCCAAAAAAGAGATCTCTATGGCTTACC
CATACGATGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGCCCGGGGTATCCG
AATTTCGCGGCCGCGTTCGACGCGCTCCCCGGCGGAGATGAGGAACTGAGGCTCAGAGAGGTCACA
TGGCTTGCTCGAGGCCCCCAGCCAGTAAGTGTGGACCCGACATTCGCACTGGCACCTGATACGC
TGTTCTGACCATGGCGTTCTCAGAGCAGGACCGTGACGGGCTGACCGGGGAACCGTAGTCCTG
GCTGAGGGCACTGCTCAGGCAAAGGTGTGACCCCAACAACCTGCCCCCGGGCCACCTGCCGGAC
GCTGGCCTCTACCCTTCAGCAGACGCCGGAGAGAGATGAGTAGCAACAAAGAGCAGCGGTCAGCA
GTGTTTGTGATCCTCTTTGCCCTCATCACCATCCTCATCCTCTACAGCTCCAACAGTGCCAACGA
GGTCTTCCACTACGGCTCCCTGCGGGGCCGCACGCGTCGGCCAGTCAACCTCAAGAAGTGGAGTT
TTTCCAGCGCCTACTTCCCTTATCCTCGGGCAACAAGACGCTTGCCGTCCAGGGGCAANCCAGTG
TGNTGATCATTACCAGCTTCCAGCCACNTGCTTGGGGCACNAAAACCTGGGGCCCTNGAGATTTG
AGGCGGGGGTTGAGTGGCACCATTCCGCNATGAACGATGGCTTCCACCTCNTNGGGTTACTNCN
GGCTGAANGTCTGGNAAACAAAACCNNCCTT

Creatine kinase

TCTTTTCGATGATGAAGATACCCACCAAACCCAAAAAAGAGATCTCTATGGCTTACCCATACGA
TGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGcCcCGGGGATCCGAATTCGCG
GCCGCGTCGACACCAGATCCTGCTCTGAATCTTTGTAGCCGCATCCCCAAGCCATGGCTGGTCCC
TTCTCCCGTCTGCTGTCTGCCCGCCCTGGACTCAGGCTCCTGGCTTTGGCTGGAGCTGGGTCTCT
CACCGCCGGGATTCTGCTCCGCCCGGAATCTGTAGGAGCTGCCGCTGCTGAACGGAGGAGACTGT
ATCCCCCGAGCGCTGAGTACCCAGACCTNNTTNNNNCACNACAACNGCNTGGCCAGTCACCTGAC
TCCAGNAGTCTATGCACGGNTCTGCGTACAAAGAACNACACCCACTTGGGTGGACATCTAGAAT
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TTGTGGGGTCNANGGNTGGGCTGGGATNNATGAAGGAAGCACCTNCTGCAGGGTAATTCTTNGC
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AACTGTCCCNAAGTATATAAAGATTTTCNCGATTTTCTGTGGNTTTATCCTTTTTGNAATGCAANC
ANGTNTANTGNNATTTTGGNTCTTTCNCACA

Protein kinase A- β

TTTAAAAGGAAAGCTACATGTTGCTTTTTGTAGTTTTGAGCAATATTTTTGCTGACCAGTGGCTT
GAAGATAAACTCTCTAATGATTATTTTTATTTTGTAGTAGCTTGAATTCAGTTTTGCCAAAACCTCT
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GGGGTCACTGATGCAGCAGTGAGTCAGAGCGCCTAGAGAGCAGTGAGGACTCCTGGACGAGAGGC
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GAGGGGCATGGCTCCTCGCCGCAGCTGTCTCCCTTGGTACCGACCTCTCTGTGGGCTGGAGAGAG
GCCTAAGGTTTTCTGAGCCTTGGCTCTTCCATGGGGGCTTCCAGCCAGTATTTGAGGAAAGTGTGAC
TGAGAAATGACACTTTGGGAGGTTTTCTCCGTCTCATCGGTGGGCTCTTAAGGGAAAAGAGCAAA
CGTGCAATTTTGGATAANTGCTAAGTCAGCAGAAGCCTTGATACCTGCTTGCCAGCTTTANAGGTT
CTCTCCTTTAAAGGGAACCTTGNTCTGTTTGTGCTTTGGGTTTCGNACCAGGTTAGAAAACAACTTGT
CTGTTTGTGCTTTGGTTCGTACCAGGTAAGAAAACA

TANK-binding kinase

TCTNTTCGATGATGAANATCCCCACCAAACCCAANAAAAGAGATCTCTATGGCTTACCCATACGA
TGTTCCAGATTACGCTAGCTTGGGTGGTCATATGGCCATGGAGGCCCCGGGGATCCGAATTCGCG
GCCGCGTCGACCGAAGCCGGAAGTAGCCTGGGGCGCGAGAAGGCCCGGGAGCCGCGGGCTGTACG
CGGCGGACACTCGCGGGCATAACATGCAAATCTCTTCTTCCCCCTTATCGTGAGGAGAAGCGCCTG
GACAAGCCGAGATGCAGAGCACCTCCAACCATCTGTGGCTCCTGTCTGATATCCTAGGCCAGGGG
GCCACTGCAAATGTCTTCCGAGGAAGGCATAAGAAAACCTGGTGATCTCTATGCTGTCAAAGTATT
TAATAACATAAGCTTCCCTTCGCCCAGTGGATGTTCAAATGAGAGAATTTGAAGTGTTAAAAAAC
TCAATCACAAAAACATTGTCAAGTTATTTGCTATTGAAGAGGAGACAACAACAAGACATAAAGTG
CTTATTATGGAGTTTTGTCCCTGTGGGAGTTTATACACTGTTCTAGAGGAGCCGTCCAATGCGTA
TGGACTTCCAGAATCAGAATTTCTCATTTGTCTTACGAGATGTGGTGGGCGGGATGAATCATCTCC
GAGAGAACGGCATAGTGCACCGAGATATCAAGCCAGGGCAACATCATGCGCGTCATAAGGGNAGG
ACGGCCAGTCTGGTGTACAACTCACGGGATTTTCGGCGCCGCTCGAAGAgCTGGAAGgANNNATG
AgCAgTTTTGTGTCTCTTGTA

Curriculum vitae*Laura Elena Paraoanu***Personal data:**

Date of birth	May 10, 1977
Place of birth	Piatra Neamt, Romania
Parents	Valeria Paraoanu, born Ciocan and Ioan Paraoanu
Citizenship	Romanian
Civil status	Single, no children

Education and Practice:

1983-1987	Primary School in Piatra Neamt, Romania
1987-1991	Secondary School in Piatra Neamt, Romania
1991-1995	Gymnasium: Petru Rares Liceu, Piatra Neamt, Romania
1995-1999	Study of biology at the University of Bucharest, Romania, specialization biochemistry Diploma work in the group of Prof. Dr. Flonta, Department of Biophysics, on 'Characterization of the insertion of quercetin in artificial lipid bilayers'
1999-2000	Master of Science study, Specialization neurophysiology and biophysics Student in the frame of Sokrates Cooperation Programme, 6 months practical work in the Department of Developmental Biology and Neurogenetics, TU Darmstadt (04/2000-09/2000)
Dec, 2000 - June, 2004	PhD-thesis in the group of Prof. Dr. P.G. Layer at the University of Technology, Darmstadt on 'Binding partners for mouse acetylcholinesterase in the CNS' <ul style="list-style-type: none">• Supported by a DAAD scholarship (2001-2004)• Laboratory work at the University of Heidelberg in the group of Dr. Uwe Ernsberger (2001)• Laboratory work at the University of California, San Diego in the group of Prof. Dr. Palmer Taylor (2002)• Assistance in practical student courses in animal physiology and developmental biology

List of publications and manuscripts

Bytyqi, A.H., Paraoanu, L.E., Duysen, E.G., Lockridge, O., Layer, P.G. (2002) Early distortion of network formation in the retina of an AChE knockout mouse. VIIth International Meeting on Cholinesterases, Pucon, Chile.

Paraoanu, L.E., Layer, P.G. (2003) Binding partners for AChE in the CNS. Xth Göttingen Neurobiology Conference, Göttingen, Germany.

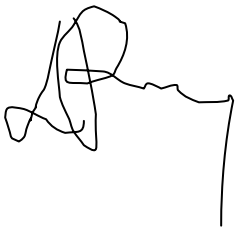
Paraoanu, L.E., Layer, P.G. Mouse acetylcholinesterase binds to laminin-1 in vivo. (submitted)

Paraoanu, L.E., Layer, P.G. AChE and Neurexin-1 β expression patterns in the developing chicken retina. (in preparation)

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Hilfsmitteln angefertigt habe.

Darmstadt, den 13.05.2004

A handwritten signature in black ink, consisting of a series of loops and a long vertical stroke at the end.

Laura Elena Paraoanu